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PHYSIOLOGICAL ZOÖLOGY

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OBSERVATIONS ON HYDRA, WITH SPECIAL REFERENCE TO ABNORMAL FORMS AND BUD FORMATION[1]

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THE present paper aims to present observations on hydras during six years' cultivation in the laboratory and to compare them with the observations of others (Parke, 1900; Grosz, 1925; Hyman, 1928). Surprisingly, tentacle formation and the determination of tentacle number have not been fully investigated previously. New problems attacked have been the formation, addition, and resorption of tentacles and the dependence of tentacle number on size, age, and other factors. Tentacle increase through treatment with chemicals will be reported elsewhere.

The species used was designated *Hydra oligactis* Pallas 1776 in previous publications but is now believed to be an undescribed species.

CULTURE METHOD

The animals were collected from submerged vegetation in ponds and streams in the vicinity of Yenching University, Peking. Tap water was conditioned by standing for 2 or 3 weeks with *Ceratophyllum* and other aquatic plants. The water is suitable when hydras placed in it expand fully; in unsuitable water the animals remain more or less contracted. After the water has been conditioned, the presence of aquatic plants is unnecessary, but in large stock cultures small amounts of plant material are helpful in adding oxygen and reducing the necessity of frequent change of water. Accumulated bottom debris is removed at intervals, and small amounts of suitable water are added from time to time. Room temperatures in which the cultures were kept ranged from 14° to 20° C. in winter, 18° to 22° C. in spring and autumn, and 23° to 25° C. during the summer months; when temperatures in summer rose above 25° C., the cultures were transferred to a wooden icebox. The cultures were kept covered.

Several species of *Daphnia* were used food daily or every other day. *Daphnia* is the ideal food because of its slowness, constant activity, and weak resistance. *Daphnia* was mostly obtained from a goldfish keeper who collected them from stagnant ponds near Peking; during the winter it was reared in the laboratory in large goldfish basins on Bond's yeast medium or soil-water extract.

DEPRESSION

The greatest difficulty in the continuous culture of hydras is the occurrence of depression. In spite of every care, cultures may pass into this state at intervals and will die out, unless prompt

[1] [This article has been extensively edited by Dr. L. H. Hyman.—EDITOR.]

measures are taken. In depression, column and tentacles fail to expand, the animals cease to feed, shorten to a stumpy appearance, and finally gradually disintegrate from the tentacle tips aborally. The most successful procedure for reviving depressed hydras is transfer to fresh suitable water; lowering the temperature is also helpful.

The histological changes occurring during depression have been fully described by Rehm (1925). During depression, entodermal cells loosen and pass into the coelenteron; the mesogloea then dissolves, and the ectodermal cells disintegrate in the same manner as the entoderm. These changes progress basipetally.

Various causes of depression have been suggested by previous workers as: long-continued rich feeding (Frischholz, 1909; Rehm, 1925); lack of oxygen or fouling of the water (Frischholz; Rehm; Goetsch, 1922; Grosz, 1925); extremes of temperature (Goetsch; Grosz); transfer to clear fresh water (Frischholz; Goetsch); infestation with protozoan symbionts (Grosz). Our experiments indicate that none of these factors causes depression when acting singly. Our results may be briefly summarized as follows:

1. EXTERNAL CAUSES

Under this head may be considered all environmental factors, such as food, temperature, population density, and physical and chemical conditions in the culture water.

a) *Food.*—Continued rich feeding does not induce depression if the cultures are kept at low temperature or if the water is changed frequently; but it may cause depression at high temperature (above 30° C.) or under infrequent change of water. The combination of these conditions perhaps brings on depression by fouling the water.

b) *Temperature.*—High temperature (above 30° C.) does not induce depression if the water is changed every other day and debris promptly removed. Under such care, cultures continued in healthy condition for 3 months without depression at high temperature. The animals budded very rapidly (three buds every 2 days) and consequently reduced to a small size, with usually less than six tentacles. Upon return to a lower temperature (20° C.), the hydras usually grow in size, sprout additional tentacles, cease budding, and pass into the sexual state. Thus change from a higher to a lower temperature does not cause depression, but transference from a lower to a higher temperature may do so, probably only in conjunction with fouling of the water.

c) *Condition of the water.*—In our opinion, this is the most important factor concerned in depression but is also the most difficult to analyze. Traces of heavy metals (Cu, Hg, Ag) will result in a disintegration resembling the disintegration of depression, whereas many other chemicals kill without disintegrative action. Narcotics (chloretone, sodium barbital, procaine hydrochloride) produce disintegrative changes in some cases and not in others; 1 per cent alcohol causes disintegrative changes most nearly resembling those of depression. Some substances (atabrine, parathion) inactivate the animals without killing them, although often destroying their tentacles; such animals may live a month or so and recover on return to normal pond water.

Fouling of the culture water results from the decaying of ejected daphnids and the accumulation of waste products and excreta; these factors may act on the hydras by producing toxic substances. Thus it is well known that urea inhibits the growth and metamorphosis of tadpoles. Other factors that induce depres-

sion may be referred to water fouling. Thus high temperature accelerates decay and accumulation of possible toxic substances. Rich feeding also may act by increased production of excreta and waste products. As will appear shortly, high population density is also a factor in depression and likewise may act through increased production of toxic substances.

Transfer to clear fresh water, such as freshly drawn tap water, very often induces depression. Although this might result from the presence in the water of minute traces of minerals, this explanation seems improbable, since the conditioning of such water makes it suitable for hydras. What happens when water is conditioned has not been analyzed for hydras, but a partial analysis in regard to fish has been made by Allee, Bowen, Welty, and Oesting (1934). Evidently, the addition of plant material to water adds something necessary for the well-being of hydra.

d) *Population density.*—A newly started culture never develops depression, and cultures with small numbers of animals rarely do so. Depression commonly occurs in crowded cultures and may result from increased excretion of waste products with increase in the population or from factors associated with the aging of the culture. The fact that depression occurs most frequently in densely populated cultures gave rise to an early suggestion, presumably erroneous, that depression is a contagious disease.

2. INTERNAL FACTORS

The analysis of the effect of internal factors on depression offers great difficulty. Here may be considered age, size, and physiological state.

a) *Age.*—According to Hyman (1928), a condition of senescence (physiological, not necessarily related to age in time) is the primary cause of depression. "Senescence is primarily a decrease in rate of dynamic processes conditioned by the accumulation, differentiation and other associated changes of the material of the colloid substratum" (Child, 1915). Depression, according to Hyman, ensues when physiologically senescent animals are subjected to slightly unfavorable conditions. "In support of this view are the facts that young animals and regenerated animals are less likely to undergo depression than ordinary large animals." According to our observations, newly detached young hydras will undergo the typical disintegrative changes of depression when transferred to a culture in depression. Nevertheless, we are in accord with Hyman's view.

b) *Size.*—There is no correlation between size and depression. We were under the impression that small hydras are more liable to depression than large ones, but analysis of the data reveals that large hydras require a longer period to pass through the disintegrative changes and hence are more readily saved.

c) *Physiological state.*—Undoubtedly, the physiological state of the hydras is the primary cause of depression. Hyman (1928) has shown with exposure to cyanide that depressed specimens are generally in a lower metabolic state than normal specimens starved for the same length of time. We have found that treatment with very low concentrations of phosphate will save depressed animals about as well as transfer to conditioned water, but growth after the phosphate treatment is greatly accelerated. The bearing of this fact on the relation of depression to physiological state is not explicable at present.

In general, we conclude that the causes of depression are the physiological state and the condition of the culture water;

other factors act by influencing these two factors.

BUD AND COLONY FORMATION

1. THE BUDDING ZONE

It is well known to all observers of hydra that these animals normally bud at a definite region, namely, the body region just distal to the stalk. The stalk almost never buds (we did observe one outgrowth on the stalk that later became a pedal disk), and therefore the budding zone is aborally limited. Orally it is not definitely limited, and buds may be caused to grow quite near the peristome by treatment with narcotics. However, one may say that the oral fifth of the column never bears any buds. Hyman (1928) has pointed out that the budding zone is not altogether fixed but is subject to physiological alteration. She reported finding one *H. oligactis* in nature with two budding zones, and she followed over a long period the history of a male culture of *H. americana* that budded irregularly as a result of a chronic depressed condition.

2. BUD ARRANGEMENT

It is also well known to observers of hydra that successive buds arise in a spiral that ascends orally, as shown schematically in Figure 1. The first bud is always formed near the aboral end of the body, and succeeding buds arise in a more and more oral direction from this. Buds form only anterior to pre-existing buds, never posterior to them. The spiral arrangement may not hold strictly for the most distal buds, if the number of buds exceeds five, for these may be somewhat irregularly arranged.

The position of the bud on the parent-body is not fixed, but, as the oldest buds detach, the next in succession shift aborally to the level (but not the position) of the former. The rate of this migration varies with the rate of budding and the length of the parent-body. At 20° C. with about one bud formed daily, the migration rate is about 1 mm. per day. As far as we are aware, this migration has not previously been recorded.

3. NUMBER OF BUDS PER HYDRA

Hydras collected from nature usually have only one or two buds, rarely three. In laboratory cultures, however, hydras may bear as many as eight buds, the oldest often with secondary buds (Figs. 2, 3).[2] Hyman (1930) and Turner (1950) reported *H. oligactis* with five and six buds as of common occurrence in flourishing laboratory cultures, and Turner found that this species might bud at intervals of about 5 hours under maximum conditions. Such many-budded hydras could be regarded as temporary colonies, although they do not seem really comparable to the long-lasting colonies observed by Hyman (1928). In our cultures a temporary colony resulted when a longitudinally dividing hydra delayed the final separation at the foot, while each component continued to bud (Fig. 7).

4. BUDDING AND GONAD FORMATION

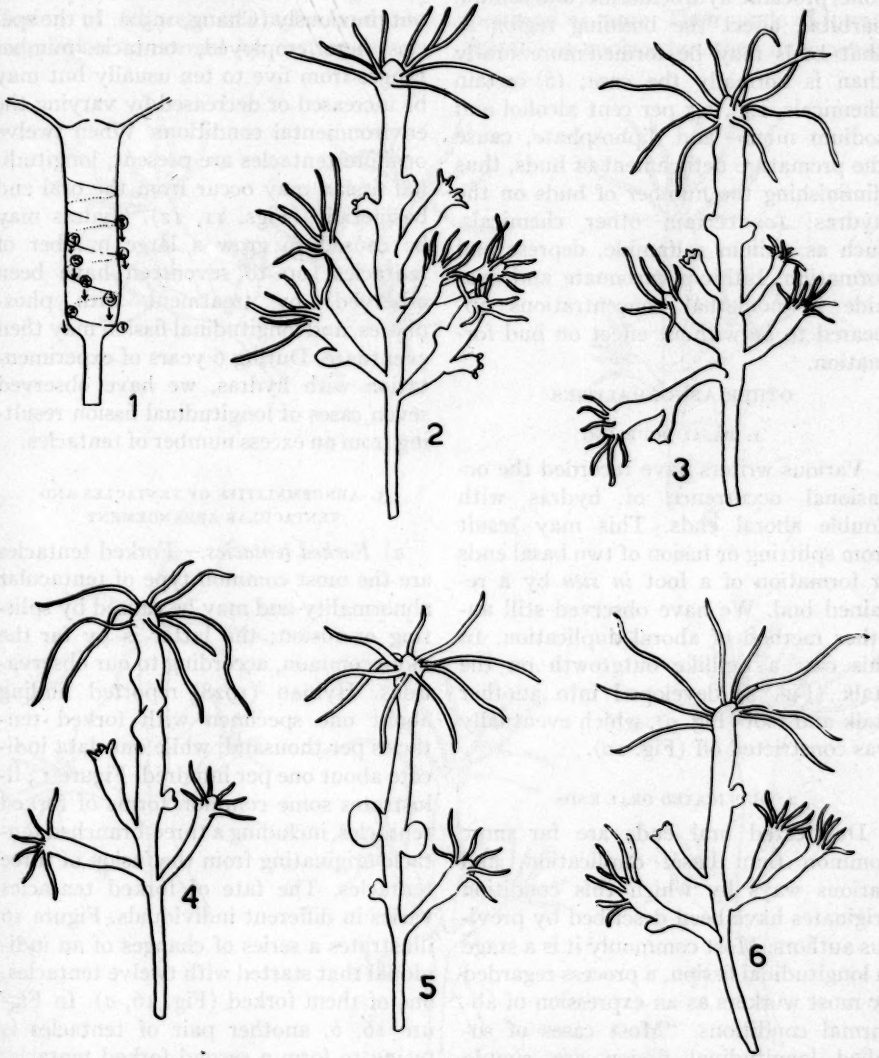
Confirming previous authors, we find that gonad formation has no influence on budding. Figure 4 shows a budding hydra with testes and Figure 5 one with ovaries.

5. TWIN BUDS

Twin buds are not uncommon, occurring at the rate of about one per thousand. Twin buds arise (*a*) by the fusion of two buds located in close proximity

[2] [It was impossible to ascertain from the authors whether the wide spacing of buds in Figs. 2 and 3 is normal or whether these figures are schematized. They appear to be natural; if so, the wide spacing of buds differs markedly from anything seen in other hydras by Dr. Hyman in an extensive experience with these animals.—EDITOR.]

and (*b*) by the longitudinal division of a bud, observed only once. A hydra with a twin bud is illustrated in Figure 6. Twin buds complete division either before detachment from the parent or after detachment. This kind of longitudinal division is independent of the number of tentacles, whereas, normally, longitudinal division occurs only in case of excessive tentacle number (Chang, 1950).



FIGS. 1–6.—Fig. 1, diagram to show the spiral arrangement of successive buds; the arrows indicate aboral migration of buds after detachment of bud 1. Figs. 2 and 3, hydras with seven or eight buds and secondary buds. Fig. 4, male hydra with testes and buds simultaneously. Fig. 5, female hydra with two ovaries and two buds. Fig. 6, hydra with twin bud.

6. ACTION OF CHEMICALS ON BUDDING

This topic will be reported in more detail later. The results obtained may be summarized as follows: (*a*) certain chemicals, chiefly narcotics, such as chloretone, procaine hydrochloride, and sodium barbital, affect the budding region so that buds may be formed more orally than is normally the case; (*b*) certain chemicals, e.g., 2.5 per cent alcohol and sodium mono- and diphosphate, cause the premature detachment of buds, thus diminishing the number of buds on the hydras; (*c*) certain other chemicals, such as sodium sulfamide, depress bud formation. Lithium carbonate and cyanide in nonlethal concentrations appeared to be without effect on bud formation.

OTHER ABNORMALITIES

1. BASAL DOUBLING

Various writers have recorded the occasional occurrence of hydras with double aboral ends. This may result from splitting or fusion of two basal ends or formation of a foot *in situ* by a retained bud. We have observed still another method of aboral duplication. In this case a budlike outgrowth on the stalk (Fig. 8) developed into another stalk and foot (Fig. 9), which eventually was constricted off (Fig. 10).

2. DUPLICATED ORAL ENDS

Duplicated oral ends are far more common than basal duplication, and various ways by which this condition originates have been described by previous authors. Most commonly it is a stage in longitudinal fission, a process regarded by most workers as an expression of abnormal conditions. "Most cases of so-called longitudinal fission are simple basipetal splittings of double-headed animals which have arisen through recovery after depression" (Hyman, 1928). According to our findings, longitudinal fission also may ensue in specimens with an abnormally large number of tentacles (over twelve).

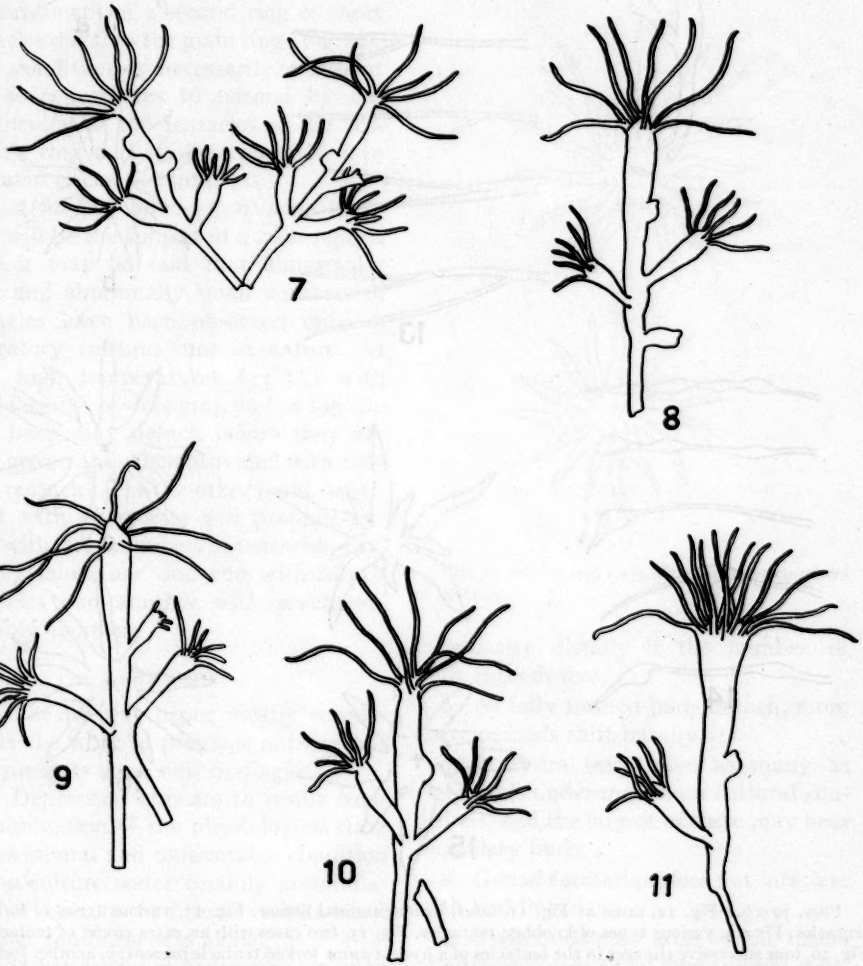
The relationship of tentacle number to longitudinal fission has been pointed out previously (Chang, 1950). In the species here employed, tentacle number ranges from five to ten usually but may be increased or decreased by varying the environmental conditions. When twelve or more tentacles are present, longitudinal fission may occur from the oral end basipetally (Figs. 11, 12). Hydras may be caused to grow a large number of tentacles (up to seventeen have been observed) by treatment with phosphates, and longitudinal fission may then eventuate. During 6 years of experimentation with hydras, we have observed seven cases of longitudinal fission resulting from an excess number of tentacles.

3. ABNORMALITIES OF TENTACLES AND TENTACULAR ARRANGEMENT

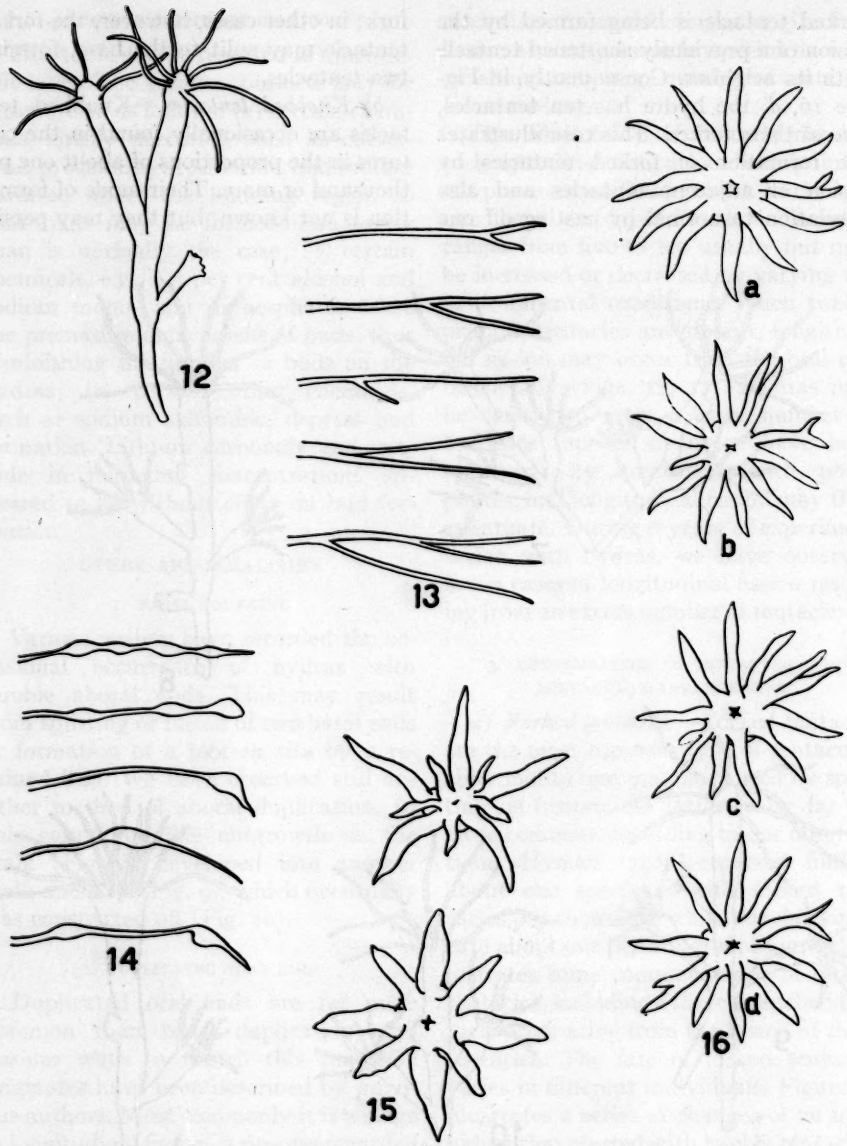
a) *Forked tentacles.*—Forked tentacles are the most common type of tentacular abnormality and may be caused by splitting or fusion; the latter is by far the more common, according to our observations. Hyman (1928) reported finding about one specimen with forked tentacles per thousand, while our data indicate about one per hundred. Figure 13 illustrates some common forms of forked tentacles, including a three-branched tentacle originating from the fusion of three tentacles. The fate of forked tentacles varies in different individuals. Figure 16 illustrates a series of changes of an individual that started with twelve tentacles, one of them forked (Fig. 16, *a*). In Figure 16, *b*, another pair of tentacles is fusing to form a second forked tentacle. In Figure 16, *c*, the original forked tentacle has reverted to normal by constricting off one of the forks, but another

forked tentacle is being formed by the fusion of a previously shortened tentacle with its neighbor. Consequently, in Figure 16, *d*, the hydra has ten tentacles, two of them forked. This case illustrates the formation of forked tentacles by fusion of adjacent tentacles and also regulation to normal by casting off one fork; in other cases, however, the forked tentacle may split to the base, forming two tentacles.

b) *Knobbed tentacles.*—Knobbed tentacles are occasionally found in the cultures in the proportions of about one per thousand or more. Their mode of formation is not known, but they may persist



FIGS. 7–11.—Fig. 7, temporary colony formed by a hydra undergoing longitudinal fission not yet quite complete. Fig. 8, hydra with budlike outgrowth on stalk. Fig. 9, same, bud has developed into a stalk and pedal disk. Fig. 10, same, accessory foot constricted off. Fig. 11, hydra with excessive number of tentacles (13).



FIGS. 12–16.—Fig. 12, same as Fig. 11, later, in longitudinal fission. Fig. 13, various types of forked tentacles. Fig. 14, various types of knobbed tentacles. Fig. 15, two cases with an extra circlet of tentacles. Fig. 16, four successive changes in the tentacles of a hydra: *a*, one forked tentacle present; *b*, another forked tentacle forming, one tentacle has shortened; *c*, original tentacle has regulated to normal by constricting off one fork, short tentacle has fused with adjacent tentacle; *d*, hydra now has two forked tentacles.

for a long time, although they eventually disappear. Several forms of knobbed tentacles are illustrated in Figure 14, of which the second from the top is the most common.

c) *Two-circleted condition.*—This abnormality is very common in early spring and late fall in laboratory cultures with good feeding and consists in the outgrowth of a second ring of short tentacles distal to the main ring (Fig. 15). This condition is necessarily transient and soon regulates to normal by the lengthening of the tentacles of the secondary ring and its incorporation into the main circlet of tentacles.

d) *Abnormal numbers of tentacles.*—This will be the subject of a later report. Here it may be said that abnormally large and abnormally small numbers of tentacles have been observed only in laboratory cultures, not in nature. At very high temperatures (33° C.) with good feeding, hydras may bud so rapidly that buds may detach before they are fully grown and when provided with only four tentacles. On the other hand, treatment with phosphate will produce hydras with large numbers of tentacles. Figure 17 shows one oral end with fifteen tentacles and another with seventeen, possibly eighteen.

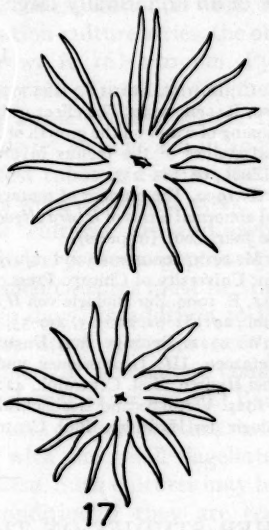


FIG. 17.—Two oral ends with excessive numbers of tentacles.

SUMMARY

1. The present paper mostly supplements the work of previous authors but also presents some new findings.

2. Depression appears to result from a combination of the physiological state of the animal and unfavorable condition of the culture water (mainly accumulation of excreta and toxic substances). Neither condition acts alone.

3. Other causes of depression assigned by previous workers, such as high temperature, excessive feeding, and so on, are not effective when applied singly but may act secondarily by affecting the causes stated in paragraph 2.

4. The regular budding zone of hydras is limited posteriorly by the distal end of the stalk, but its anterior limitation is subject to physiological alteration.

5. The well-known spiral arrangement of successive buds tends to some irregularity distally if the number of buds exceeds five.

6. As fully formed buds detach, more anterior buds shift basally.

7. A hydra may have as many as eight buds under maximum cultural conditions, and the largest of these may bear secondary buds.

8. Gonad formation does not interfere with budding.

9. Twin buds may arise by the fusion of adjacent buds or by longitudinal splitting of an originally single bud.

10. Budding may be affected by certain chemicals; some depress budding,

others cause premature detachment of buds, and still others may influence the length of the budding zone.

11. A case was observed of basal duplication by a budlike outgrowth from the stalk.

12. Apical duplication may be caused by longitudinal division, which may be initiated, among other causes, by the presence of an abnormally large number of tentacles (more than twelve).

13. Cases of forked and knobbed tentacles are presented.

14. Double tentacular circlets may arise by the formation of a second ring of small tentacles within the old ring; regulation to one circlet eventuates.

15. Deviations from the normal tentacle range of five to ten may be produced in laboratory cultures.

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FURTHER STUDIES ON THE STERILE CULTURE OF PARAMECIUM

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In 1942 Johnson and Baker first reported the sterile culture of *Paramecium multimicronucleata*. This was accomplished by using one part of sterile pressed yeast juice (p.y.j.) in 10 parts of sterile triple-distilled water. Later it was found that better growth could be obtained by using Bacto proteose-peptone medium—1 part p.y.j. to 20 parts of 0.5 per cent proteose-peptone. The best growth obtained in this medium was one division every 48 hours.

In 1945 Johnson and Tatum found that there are two fractions of p.y.j. which are essential for the growth of *P. multimicronucleata*. One fraction is heat-labile. When the sterile p.y.j. is dialyzed for 18 hours against equal volumes of distilled water and the two fractions are tested for growth, the dialyzate has no activity, while the undialyzable fraction gives good growth. When the p.y.j. is exhaustively dialyzed against running distilled water, the undialyzable fraction gives no growth when added to the proteose-peptone medium. However, good growth is obtained if a small amount of autoclaved p.y.j., from which the coagulated proteins have been removed, is added to the proteose-peptone

medium containing the undialyzable fraction. The autoclaved fraction alone will not support growth, nor will the undialyzable fraction support growth after autoclaving, either alone or in combination.

The heat-labile fraction can be precipitated from the p.y.j. at three-fourths saturation with ammonium sulphate. The precipitated fraction, when dissolved in distilled water, dialyzed, and filter-sterilized, gives growth when added to the proteose-peptone medium containing autoclaved p.y.j. When this material is purified by three repeated precipitations with ammonium sulphate, it gives good growth. The precipitated fraction coagulates upon heating and will not support growth after autoclaving.

Van Wagtendonk and Hackett (1949) have been able to grow *P. aurelia* in sterile culture in a medium consisting of equal parts of autoclaved 0.5 per cent yeast autolysate (Basamin Busch) and a lettuce infusion in which *Aerobacter aerogenes* has grown for 24 hours and which is then autoclaved. The omission of any of the three ingredients—yeast autolysate, lettuce infusion, bacteria—resulted in no growth. Thus they were able to grow *P. aurelia* in a medium with only heat-stable factors.

The medium in which cultures of *P. multimicronucleata* have been maintained for the last few years is a very complex one. Attempts were made during the past year to substitute known substances for the different constituents of this medium.

MATERIAL AND METHODS

The strain of *P. multimicronucleata* used in these studies was the same strain used in the first studies in 1942. The sterile strain of *P. aurelia* used was obtained from Dr. W. J. van Wagtendonk and Mr. C. A. Miller, of Indiana University. The p.y.j. and the various fractions were made from Fleischmann's baker's yeast as described by Johnson and Tatum (1945). The protogen used was kindly furnished by Dr. E. L. R. Stokstad, of the Lederle Laboratories. The nucleic acids used were hydrolyzed with NH_4OH according to the method of Levine and Bass (1931).

In all the experimental work, except in the isolation-culture series, the organisms were grown in 16×150-mm. Pyrex test tubes in 5 ml. of medium. All media were made in triple-distilled water. The isolation clutures were grown in 10×75-mm. Pyrex test tubes in 1 ml. of medium. All cultures were incubated at 25° C.

Stock cultures are ordinarily maintained in the proteose-peptone–p.y.j. medium. Such cultures must be transferred every 10 days. In addition to this, bacteria-free stock cultures are regularly maintained in a medium composed of equal volumes of 0.5 per cent Bacto yeast extract and 0.5 per cent dextrose which is seeded with the small flagellate *Polytomella caeca*. Such cultures may be kept in good condition if they are transferred each month. These stock cultures facilitate the work when there are long intervals between experiments. Paramecia from such stock cultures may be freed of the *P. caeca* in a few washes and then placed in the proteose-peptone–p.y.j. medium.

RESULTS

THE HEAT-LABILE FACTOR IN P.Y.J.

It has been possible to substitute hydrolyzed nucleic acid for the heat-labile fraction. Yeast nucleic acid (RNA) hydrolyzed with NH_4OH was first used. Autoclaved desoxyribonucleic acid (DRNA) gives fair growth, and DRNA hydrolyzed with NH_4OH gives as good growth as does hydrolyzed RNA. Also successful was the use of DRNA hydro-

lyzed by desoxyribonuclease (obtained from Worthington Biochemicals). Moreover, the use of a solution of the sodium salts of the nucleotides of ribonucleic acid (Schwarz) gave growth. Cultures have been carried through forty-five transfers over the last 13 months. The medium used was 1 per cent proteose-peptone containing autoclaved p.y.j. (1:20) plus 100γ hydrolyzed DRNA per milliliter of medium.

TABLE 1

ISOLATION CULTURES

Transfer	Days for Each Fission			
	Series A	Series B	Series C	Series D
1	3	2	1	2
2	2	2	2	1
3	3	1	2	2
4	1	2	2	2
5	2	2	3	2
6	2	2	1	2
7	2	3	3	2
8	3	2	2	3
9	1	3	2	1
10	2	2	3	1
11	2	2	1	3
12	2	1	2	2
13	2	3	2	2
14	1	2	3	1
15	2	2	2	2
16	1	3	3	3
17	2	2	1	2
18	2	1	3	1
19	2	3	2	2
20	1	2	1	3
21	2	2	3	2
22	2	1	2	3
23	3	3	2	2
24	1	2	3	2
25	2	2	2	.....
26	2	2	.....	.....
27	2	2	.....	.....
Av. fission rate per day	0.52	0.48	0.47	0.50

After several transfers had been made with this medium, it was suggested that a more rigorous test of the ability of *P. multimicronucleata* to stand repeated transfer in this medium should be made—that isolation cultures should be tried. The 5-ml. mass cultures are carried along by transferring 0.1–0.2 ml. of an old culture for each subculture. During the summer of 1950 several isolation cultures were started, and four of them were carried through more than twenty transfers. In these isolation cultures, one organism was isolated in 1 ml. of medium—proteose-peptone, autoclaved p.y.j., hydrolyzed nucleic acid—in a small test tube. They were examined every 24 hours. When a division had occurred, each organism was subcultured, and then the first to divide was used in the next subculture in a given series, and so on. The results of four series are given in Table 1. That this medium will support growth through many transfers is shown. Also the division rate in these cultures is the same as that observed in mass cultures.

Kidder and Dewey (1948) have reported that the purine and pyrimidine requirements for *Tetrahymena* can be satisfied with a mixture of guanylic acid and cytidylic acid. In this study guanylic acid and cytidylic acid (50γ of each per milliliter of medium) were substituted for the hydrolyzed DRNA in the culture of *P. multimicronucleata*. Cultures have now have been carried through fifteen transfers with this substitution. So far the activity of other purines and pyrimidines has not been tested.

Possibly the heat-labile fractions of the p.y.j., either the dialyzed fraction or the precipitated fraction, contain an enzyme which hydrolyzes the nucleic acid in the medium as originally used.

PROTEOSE-PEPTONE

Numerous attempts have been made to substitute for the proteose-peptone. In the beginning, Kidder's complete medium for *Tetrahymena* was used.

Several trials using this medium resulted in no growth. Kidder's medium minus the vitamins, but with the auto-

claved p.y.j. instead, also gave no growth. Negative results were obtained when hydrolyzed casein (vitamin-free) was substituted for the amino acids in the Kidder medium. Likewise, when both hydrolyzed casein and the autoclaved p.y.j. were substituted in the Kidder medium, no growth occurred. At present it has not been possible to substitute for the proteose-peptone.

wide range of concentrations, with no growth. Table 3 gives a summary of the various tests. The plus sign (+) indicates growth, the minus sign (−) no growth.

The autoclaved fraction is made by autoclaving the p.y.j. for 10 minutes, discarding the coagulum, filtering the liquid through filter paper, and then re-autoclaving. After this treatment there is always some particulate matter in it

TABLE 2

KIDDER'S COMPLETE MEDIUM FOR *Tetrahymena**

Constituents	γ/Ml of Final Medium	Constituents	γ/Ml of Final Medium
l-Arginine HCl	163	Ca pantothenate	0.10
l-Histidine	66	Nicotinamide	0.10
dl-Isoleucine	213	Pyridoxine HCl	2.00
l-Leucine HCl	247	Riboflavin	0.10
l-Lysine HCl	196	Pteroylglutamic acid	0.01
dl-Methionine	214	Biotin (free acid)	0.0005
l-Phenylalanine	110	Thiamine HCl	1.0
dl-Threonine	238	Choline Cl	1.0
l-Tryptophan	60	Protogen	0.375
dl-Valine	96	Hydrolyzed yeast nucleic acid	100
dl-Serine	317	$MgSO_4 \cdot 7H_2O$	100
l-Glutamic acid	233	K_2HPO_4	100
l-Aspartic acid	61	$CaCl_2 \cdot 2H_2O$	50
Glycine	5	$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	25
dl-Alanine	55	$CuCl_2 \cdot 2H_2O$	5
l-Proline	175	$FeCl_3 \cdot 6H_2O$	1.25
l-Hydroxyproline	75	$MnCl_2 \cdot 4H_2O$	0.05
l-Tyrosine	67	$ZnCl_2$	0.05
l-Cysteine	3.5	Tween 85	500
Dextrose	1,000		
Sodium acetate	1,000		

* Kidder and Dewey (1949).

AUTOCLAVED P.Y.J.

Johnson and Tatum (1945) reported that it was not possible to substitute a mixture of vitamins for the autoclaved p.y.j. This was repeated by using, in addition to the vitamins used in the 1945 study, pteroylglutamic acid, vitamin B_{12}, and the growth factor, protogen. Again it was not possible to obtain growth. Various yeast preparations were tried in place of the autoclaved fraction. Difco yeast extract, dried brewer's yeast and the Anheuser-Busch yeast autolysate, Basamin-Busch, were tried, each in a which partially settles out in the tubes. This fraction, when filtered through an ultra-fine Pyrex fritted-glass filter, gives no growth of the paramecia when used. It appears that the particulate matter contains something which is essential.

COMPARISON OF *P. multimicronucleata* AND *P. aurelia*

Paramecium multimicronucleata was carried through nine transfers in the yeast autolysate–lettuce infusion–bacteria medium of Van Wagtendonk and Hackett. The preparation of the lettuce

infusion used was described by Sonneborn and Dippell (1946). The division rate in this medium was the same as that referred to in the other experiments with *P. multimicronucleata*, but the cultures in this medium lasted longer. A sterile strain of *P. aurelia* was grown successfully through twenty transfers in the proteose-peptone–hydrolyzed nucleic acid–autoclaved p.y.j. medium. The growth of *P. aurelia* in this medium was slightly over 1 fission per day. In a personal communication W. J. van Wagtendonk and C. A. Miller have confirmed the growth of *P. aurelia* in the proteose-peptone–p.y.j. medium. From this it appears that the requirements of both species are essentially the same.

DISCUSSION

The substitution of nucleic acid derivatives for the heat-labile fraction of p.y.j. has solved one of the problems in the development of a medium of known constituents for the sterile culture of *Paramecium*. With the possibility of using a medium with only heat-stable components, further work will be easier. Two other problems remain to be solved—the substitution of known substances for proteose-peptone and the substitution of known substances for the autoclaved fraction of p.y.j. As mentioned above, this autoclaved fraction contains some particulate matter. When it is removed, no growth occurs. This is similar to the situation which Van Wagtendonk and

TABLE 3

VARIOUS MEDIA TESTED

No.	Constituents		Growth
1	1 per cent proteose peptone*	Filter-sterilized pressed yeast juice (p.y.j.) 1:10	+
2	" " " " "	Autoclaved p.y.j. 1:20	+
3	" " " " "	" " " Dialyzed p.y.j. 1:10	+
		Precipitated fraction of p.y.j. 1:10	
4	" " " " "	" " " Hydrolyzed RNA 100 γ/ml	+
5	" " " " "	" " " Hydrolyzed DRNA 100 γ/ml	+
6	" " " " "	" " " Cytidylic acid 50 γ/ml and guanylic acid 50 γ/ml	+
7	" " " " "	Difco yeast extract† Hydrolyzed DRNA 100 γ/ml	−
8	" " " " "	Brewer's yeast† (Anheuser-Busch) " " "	−
9	" " " " "	Yeast autolysate† (Basamin-Busch) " " "	−
10	" " " " "	Vitamin mix‡ " " "	−
11	Kidder's amino acid§ mixture plus salts	" " " " "	−
12	" " "	Autoclaved p.y.j. 1:20 " " "	−
13	Acid-hydrolyzed casein‖ (NBC) (vitamin-free) plus salts	" " " " "	−
14	" " "	Vitamin mix " " "	−
15	Bacto casamino acids‖ (vitamin free) plus salts	" " " " "	−
16	" " "	Autoclaved p.y.j. 1:20 " " "	−

* All the proteose-peptone cultures were tested, using both distilled water and the salt mixture of Kidder as given in Table 2.

† Each of these preparations was tried in a wide range of concentrations.

‡ The vitamin mix included the vitamins listed in Table 2 plus protogen. Vitamin B_{12} was added separately in certain experiments in a wide range of concentrations.

§ Each of the media listed in Nos. 11, 12, 13, 14, 15, and 16 was tested separately by adding dextrose and by adding dextrose and Tween 85 in the concentration given in Table 2.

‖ Each of these was tested in 1 and 0.5 per cent concentrations.

Hackett (1949) have described for *P. aurelia*. Whether *Paramecium* requires particulate matter per se, or whether in both these cases some essential substance is tightly bound to the particulate matter but which can be freed from it, remains to be determined in future experiments.

SUMMARY

Both hydrolyzed nucleic acid and a mixture of cytidylic and guanylic acids were substituted for the heat-labile fraction of p.y.j. in the sterile culture of *P. multimicronucleata*. Both *P. multimicronucleata* and *P. aurelia* were grown in the same medium. It was not possible to substitute mixtures of known substances for either proteose-peptone or the autoclaved fraction of p.y.j.

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STUDIES ON THE NUTRITION OF COLORLESS EUGLENOID FLAGELLATES. III. ASTASIA LONGA, A. KLEBSII, AND KHAWKINEA QUARTANA

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ALTHOUGH chemoautotrophic nutrition is known to occur in several species of bacteria, few reports have appeared concerning this nutritional method in other groups of microörganisms. Among the Protozoa two species of flagellates have been described as chemoautotrophic: *Chilomonas paramecium* (Mast and Pace, 1933) and *Astasia longa* strain J (Schoenborn, 1946). However, the conclusions of Mast and Pace have not been supported by various other workers (Hall and Loefer, 1936; Cosgrove, 1950), while additional investigations are needed with regard to *A. longa*. These more detailed studies of *A. longa* have been in progress in our laboratory for several years and will be reported later. While this work was going on, it also appeared desirable to investigate other closely related forms for their ability to carry on chemoautotrophic nutrition. In addition, an attempt was made to obtain chemoauto-

trophic flagellates from natural bodies of water. The results of these studies are reported below.

MATERIAL AND METHODS

The organism previously described as chemoautotrophic and referred to as *Astasia* sp. (Schoenborn, 1946) has been identified as a strain of *A. longa* (Pringsheim, 1948) and more recently designated as strain J (Schoenborn, 1949). A number of strains of this species were listed by Pringsheim, two of which were available for study. These two strains will be designated as follows: (1) *A. longa* strain P, which was isolated in pure culture by Pringsheim, and (2) *A. longa* strain L, isolated in pure culture and described as *A. chattoni* by Lwoff and Dusi (1934) but considered by Pringsheim (1948) to be a strain of *A. longa*. Two other species of colorless euglenoids were also available: *A. klebsii*, isolated in pure culture by Von Dach (1940), and *Khawkinea quartana*, which Pringsheim isolated in pure culture. This latter species differs from the others in the possession of an eyespot and thus belongs to the genus *Khawkinea* erected by Jahn and McKibben (1937). All these flagellates were supplied to the writer by Dr. R. P. Hall, who had received *A. klebsii* from Dr. H. Von Dach and the remaining forms from Dr. E. G. Pringsheim.

The method employed in determining whether these flagellates are capable of chemautotrophic nutrition was similar to that previously used with *A. longa* strain J. The inorganic medium contained $(NH_4)_2HPO_4$, 1.0 gm.; $MgSO_4 \cdot 7H_2O$, 0.2 gm.; KH_2PO_4, 0.2 gm.; KCl, 0.2 gm.; $FeCl_3 \cdot 6H_2O$, 0.0025 gm.; double-distilled water, 1.0 liter; HCl for pH adjustment. Serial transfers were carried out in this medium with cotton-plugged, 125-ml. Pyrex Florence flasks as culture vessels, the first transfer being inoculated from a stock culture in organic medium (Difco Bacto-tryptone or yeast extract). The first one or two transfers received only small inocula, so as to reduce rapidly the concentration of organic material carried over from the stock cultures. In later transfers the flasks received 20 ml. of medium and a 2-ml. inoculum, thereby making necessary about a tenfold increase of the initial population, during a growth period of 20–45 days, in order to maintain a constant final population density in the successive transfers. Population densities were determined as in previous work (Schoenborn, 1946). All cultures were grown in darkness at a temperature ($28° \pm 0°.5$ C.) and hydrogen-ion concentration (pH 6.5–6.9) known to be suitable for *A. longa* strain J (Schoenborn, 1947, 1949).

The importance of using pure cultures for physiological investigations of the Protozoa has been emphasized by various workers (i.e., Lwoff, 1932). However, the washing technique commonly employed to obtain Protozoa in pure culture would make impossible, within a reasonable period of time, the isolation of any large number of individuals for subsequent screening as to their chemoautotrophic ability. Hence an attempt was made to obtain pure cultures with the use of penicillin, as has been reported by other workers. Tubes of inorganic medium (prepared with spring water instead of distilled water) containing 3,000 units/ml of sodium penicillin G were inoculated with material from various streams and lakes in the vicinity of the Mountain Lake Biological Station, Mountain Lake, Virginia. After a suitable growth period these tubes were examined for the presence of both bacteria and flagellates.

EXPERIMENTAL RESULTS

Experiments with pure cultures.—Several attempts have been made to grow

each of the flagellates—*K. quartana*, *A. klebsii*, and *A. longa* strains P and L—in the inorganic medium. The results of a typical experiment are given in Table 1;

TABLE 1

GROWTH IN INORGANIC MEDIUM

Species	Transfer	Population Density (Flagellates/Ml)	
		Initial	Final
A. longa strain P...	1	265	2,607
	2	271	335
	3	35	37
	4	5	8
	5	2	0
A. longa strain L...	1	820	4,320
	2	440	1,525
	3	148	262
	4	31	32
	5	3	0
A. klebsii.....	1	1,100	3,610
	2	318	1,338
	3	122	370
	4	25	40
	5	4	0
K. quartana.....	1	4,492	4,390
	2	429	1,608
	3	155	411
	4	39	33
	5	2	7
	6	0	0

all other experiments gave similar results. It will be noted that little or no growth occurred in later transfers, when the organic material had been reduced by dilution to a small amount. This lack of growth made it necessary to discontinue the experiments after only a few serial transfers. In all these experiments *A. longa* strain J, used as a control organism, was observed to grow in each transfer, thereby demonstrating that the medium was not toxic, at least for this strain.

A number of colorless flagellates, while not chemoautotrophic, can be grown in simple solutions that contain an organic carbon source. Hence, in another series of experiments, 0.1 per cent sodium acetate was added to the inorganic solution. This medium permitted growth of *A. longa* strain J, as has been observed previously (Schoenborn, 1940, 1946), but did not permit growth of the other flagellates. In one experiment various growth factors were added to the acetate-containing solution to provide the following concentrations in gammas per milliliter of medium: pyridoxine, 10.0; nicotinic acid, 10.0; thiamine, 1.0; calcium pantothenate, 1.0; choline, 0.5; riboflavin, 0.1; folic acid, 0.0005; biotin, 0.0005. *Astasia longa* strain J was not tested in this medium. Results with the other flagellates were similar to those obtained in the inorganic solution.

Experiments to obtain pure cultures.—Before penicillin was used in attempts to establish protozoan cultures free of bacteria, experiments were carried out to determine whether this substance is toxic to *A. longa* strain J. Growth curves were obtained with varying concentrations of penicillin (0, 100, 500, 1,000, and 3,000 units/ml) added to the inorganic medium (Fig. 1). All concentrations of penicillin increased the growth rate during the

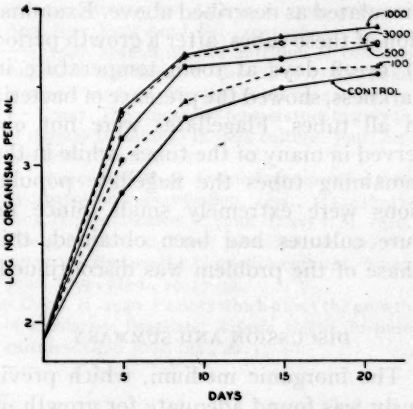

FIG. 1.—Growth in inorganic medium with penicillin added in the amounts shown (units/ml).

logarithmic phase of growth. Generation times during this phase were 38.80 hours in the control cultures, as compared to 31.72, 28.16, 24.72, and 25.11 hours for cultures with 100, 500, 1,000, and 3,000 units of penicillin/ml, respectively. A similar experiment was carried out in which the inorganic nitrogen source of the medium was replaced with Difco Bacto-tryptone. In this case the generation times during the logarithmic phase were also faster in the penicillin-containing cultures (7.72–7.98 hours) than in the controls (8.39 hours), with the fastest rate being at the 1,000-unit concentration. In both media the yields were greater in those cultures with penicillin than in control cultures.

Several experiments were then carried out in an effort to obtain bacteria-free cultures. A total of approximately two hundred penicillin-containing tubes were inoculated as described above. Examination of these tubes, after a growth period of 14–28 days at room temperature in darkness, showed the presence of bacteria in all tubes. Flagellates were not observed in many of the tubes, while in the remaining tubes the flagellate populations were extremely small. Since no pure cultures had been obtained, this phase of the problem was discontinued.

DISCUSSION AND SUMMARY

The inorganic medium, which previously was found adequate for growth of *A. longa* strain J (Schoenborn, 1946), has not supported growth of four closely related flagellates: *A. longa* strains P and L, *A. klebsii*, and *K. quartana*. The inadequacy of the medium may be due to the lack of one or more essential elements, or it may be that biochemical mutations have occurred in these flagellates, rendering them incapable of synthesizing certain necessary metabolites. This latter possibility is currently being studied, not only in these four flagellates, but in clones derived from irradiated cells of *A. longa* strain J which are likewise incapable of growth in an inorganic medium.

Astasia longa strains P and L, *A. klebsii*, and *K. quartana* also failed to grow when an organic carbon source, sodium acetate, was added to the inorganic solution. These results in the acetate-containing medium may be in conflict with those reported by Pringsheim (1948), since he states: "*Astasia longa* . . . can also multiply in a medium containing ammonium acetate and the indispensable accessory salts." Inasmuch as Pringsheim provides no details of his experiments, it is impossible to duplicate his medium or to ascertain the strains of *A. longa* that were studied. His results are not in conflict with our findings regarding *A. longa* strain J (Schoenborn, 1940, 1946), but they do differ from the results reported above for *A. longa* strains P and L.

The few reports that are available indicate that, in general, the Protozoa have a high tolerance for penicillin. Thus *Trichomonas vaginalis* was not killed by 60 hours' exposure to 1,000 units/ml (Johnson *et al.*, 1945); *Endamoeba histolytica* has been grown in serial transfer in media containing a similar penicillin concentration (Seneca *et al.*, 1949); while *Chlamydomonas pseudococcum* was found to be viable after 7 days' exposure to 75,000 units, and *Euglena gracilis* var. *bacillaris* after 4 days' exposure to 133,300 units/ml (Loefer, 1949; Loefer and Guido, 1950). As noted above, concentrations up to 3,000 units/ml are not toxic to *A. longa* strain J. In fact, growth curves indicate that this flagellate divided more rapidly during the logarithmic phase of growth and produced larger

yields in media containing penicillin than in the control media. Seneca *et al.* (1949) reported that *E. histolytica* grew slightly better in the presence of penicillin than in the controls; but, as pointed out by these authors, this may not have been a direct effect of penicillin on the amoebae, since their cultures were not bacteria-free. On the other hand, growth of *T. foetus* is inhibited by penicillin, the degree of inhibition depending on the penicillin concentration (Cole, 1947).

The relative insensitivity of Protozoa to penicillin has made possible, in some cases, the establishment of bacteria-free protozoan cultures with the use of this antibiotic (Mahmoud, 1944; Johnson *et al.*, 1945; and others). Our attempts to use this method, however, were not successful.

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NUTRITIVE REQUIREMENTS OF THE SMALL-EYED FLOUR BEETLE, PALORUS RATZEBURGI WISSMAN (TENEBRIONIDAE, COLEOPTERA)

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THE small-eyed flour beetle, *Palorus ratzeburgi* Wissman, is a minor pest of flour and stored grain (Cotton, 1941). Like *Tribolium confusum* Duval, to which it is related and which it closely resembles in appearance and food habits, it is cosmopolitan in distribution and has been reported from many sections of the United States (Chittenden, 1895). The adult beetle is mahogany-brown in color, shiny, and about 1.5 mm. in length. The larvae are white to pale yellow and may reach a size of about 4 mm. Pupae and eggs are milky-white. Pupation usually occurs within a pupal cell, which is hollowed out of the flour by the larva. The adult emerges about 5 days after pupation.

In the area around Champaign-Urbana, Illinois, this beetle has been very abundant under grain elevators, particularly in the refuse under the corn bins. Despite this apparent preference for corn, the insects could not survive in the laboratory on a diet made up exclusively of corn. Attempts were thereupon made to improve the corn diet, and this work eventually extended to a study of both natural and artificial diets in the nutrition of this insect. This paper treats of the effect of artificial diets on growth of *Palorus*, especially with reference to the vitamin requirements of this insect. The effects of various grains on *Palorus* will be discussed in another paper.

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MATERIAL AND METHODS

Experimental diets were prepared by weighing out the dry components and adding 4 gm. of the mixture to a 1 × 2-inch glass vial. This vial was fitted with a one-hole cork stopper to allow air exchange. Vitamins were dissolved in water or dilute ammonia water, pipetted directly into the vials along with added water, and the materials were mixed with a spatula. The diets were then placed at a constant temperature and humidity for at least 3 days, so that their moisture content would reach equilibrium, about 10 per cent of the dry weight.

After equilibration, the diets were ground in a mortar, and each diet was divided and placed in two vials. Ten newly emerged larvae were then added to each vial; in later experiments this was increased to fifteen and twenty without observable ill effects from overcrowding. The larger number of larvae was, therefore, used in most experiments.

Experimental vials were placed in the constant-temperature room at 30° C. and 65–70 per cent relative humidity. However, owing to mechanical difficulties, the temperature would occasionally fluctuate as much as 3° C. in either direction. These fluctuations did not appear to have any ill effect, since the presence of the diet undoubtedly served as an insulating medium that minimized any changes.

Larvae were left undisturbed on the diets for a period of about 17 days; then they were weighed. Thereafter, they

were checked periodically and, when pupation occurred, the pupae were removed and were placed in a vial on a piece of absorbent paper. Adults emerged readily under these conditions, the average pupal period being about 5 days. Some of the emerging adults had unexpanded elytra; these beetles rarely lived more than a few hours. The duration of the pupal period was not affected by the food material used.

Artificial diets were employed in order to determine the vitamin requirements of the insect. The basic diet used consisted of the following:

	Parts
Casein (vitamin-free, Labco)	20
Glucose (anhydrous)	80
Cholesterol	1
Salts (McCollum-Davis No. 185)	2
Water	10

This mixture was the same basic diet as that described by Fraenkel and Blewett (1947) for *Tenebrio molitor* L. The casein and salts were mixed together first, with water to dissolve the salts, and the mixture was ground up when dried. Cholesterol was then ground in, followed by the glucose. Grinding was done in a mortar.

To this diet was added dried brewer's yeast or the vitamin mixture to be tested. Three μgm. of a B_T concentrate (Fraenkel *et al.*, 1948) per gram of the dry diet were routinely added to all artificial diets lacking a source of this factor. The B_T concentrate used was one that was standardized by bioassay with *Tenebrio* (Fraenkel, 1951), the same concentrate being used in all experiments described herein. The B_T concentrate was added to half the water in the diet, and an "all-vitamins" mixture was dissolved in the other half to complete the diet. The all-vitamins mixture was made up of the following vitamins:

Vitamin	μgm/gm of Diet
Thiamin	25.0
Riboflavin	12.5
Nicotinic acid	25.0, later 50
Pyridoxin	12.5
Calcium pantothenate	25.0
Choline chloride	500.0, later 1,000
i-Inositol	250.0
Folic acid	2.5
(Biotin	0.5, omitted at times)

In the early experiments, weighing of the larvae was done when the first pupal cell was discovered, but, since this time criterion was open to too great a possible error, a period of about 17 days was adopted. It was found that larvae on a good diet would reach maximum weight at about 17 days and would soon be ready to form prepupae. Larval weight thus obtained served as an indication of the rate of larval growth on the test diet. Average time for pupation is a measure of the total length of the larval period. The number of adults that emerged from pupae on a particular diet can be taken as a measure of the completeness of the diet.

EXPERIMENTS

PROTEIN AND CARBOHYDRATE REQUIREMENTS

Palorus proved to require a diet rich in carbohydrates. By trying various proportions of protein and carbohydrate, the optimal mixture was found to be 20 per cent casein and 80 per cent glucose. Cornstarch in proportions of 50–80 per cent was similar in its effect to glucose at 80 per cent, starch at 20 per cent being somewhat better than an equal amount of glucose. This may be due to the extremely small size of the cornstarch particles, enabling a greater consumption by the larvae than would be possible with glucose. Owing to the chemical complexity of starch, however, glucose was exclusively used in basic diets. Cholesterol, salts, water, and yeast made up the re-

mainder of the diet. Table 1 shows the effect of various proportions of carbohydrate and protein and also the effect of the lack of cholesterol and yeast.

Growth was very poor without carbohydrate, but apparently a protein was not needed. This is explained by the presence of yeast, which, at a concentration of 5 per cent, appears to contain sufficient protein for *Palorus*. The yeast also provides ergosterol, so that the "no-cholesterol" diet was not actually devoid of a sterol. A better indication of the need for cholesterol may be noted from Table 2. Larvae were unable to grow in the absence of yeast, and no pupae were formed.

TABLE 1

EFFECT OF VARIOUS PROPORTIONS OF PROTEIN AND CARBOHYDRATE ON THE GROWTH AND DEVELOPMENT OF *Palorus*

Diet*	Larvae at 22 Days		Pupation		
	No.	Weight (Mg.)	No.	Av. Time (Days)	Spread (Days)
Casein 100	17	0.34	7	43.7	38–50
Casein 80, glucose 20	14	0.52	8	40.6	36–50
Casein 50, glucose 50	18	1.01	14	31.7	30–40
Casein 20, glucose 80	20	1.10	20	27.9	26–30
No casein, glucose 100	17	1.17	16	28.9	26–31
Casein 80, starch 20	19	1.14	11	30.2	29–31
Casein 50, starch 50	20	1.21	18	27.3	26–29
Casein 20, starch 80	19	1.19	17	27.3	26–29
No cholesterol	20	0.97	13	31.5	30–38
No yeast	16	0.11	0	.....	.....
No yeast or cholesterol	16	0.08	0	.....	.....

* Diets contained salts 2 parts, water 10 parts, brewer's yeast 5 parts, cholesterol 1 part, casein 50 parts, and glucose 50 parts unless otherwise designated.

By leaving individual vitamins of the B complex out of the diet of *Palorus*, most of them were found essential for this insect. The required vitamins included thiamin, riboflavin, nicotinic acid, pyridoxin, calcium pantothenate, choline chloride, and B_T, while biotin, folic acid, and *i*-inositol seemed to have little or no effect. This is shown in Table 2. Growth in the absence of biotin and folic acid may have been due to the presence of these substances in the casein used, since only a very slight amount of these vitamins is ordinarily necessary for an effect. Inositol has never been shown to be needed by insects (Fraenkel *et al.*, 1950).

Tribolium confusum, as stated above, is closely related to *Palorus* and has been investigated before for its vitamin requirements (Fraenkel and Blewett, 1943, 1947). It was therefore decided to run an identical experiment with *Tribolium* (Table 3) simultaneously with the one summarized in Table 2. Since *Tribolium* does not need B_T, it was omitted from the diets. This experiment shows that, in

GENERAL VITAMIN REQUIREMENTS

Various attempts to determine the vitamin requirements of this insect were unsuccessful until it was learned that vitamin B_T (Fraenkel *et al.*, 1948) was necessary for normal growth. Thereafter, a concentrate of B_T was routinely added to the artificial diets.

contrast to *Palorus*, *Tribolium* does not need choline, whereas lack of biotin has some effect on *Tribolium*. Contrary to earlier observations (Fraenkel and Blewett, 1947), *Tribolium* did not suffer from the lack of folic acid, which would suggest that the casein in this experiment might have contained impurities of this vitamin.

Tenebrio molitor, the mealworm, is an-

TABLE 2

GROWTH AND DEVELOPMENT OF *Palorus* ON DIETS DEFICIENT IN INDIVIDUAL B VITAMINS

Addition to Basic Diet*	Larvae at 17 Days		Pupation			Adult Emergence	
	No.	Weight (Mg.)	No.	Av. Time (Days)	Spread (Days)	No.	Per Cent
Yeast (5 per cent)	21	0.80	13	34.0	22–39	10	78
All vitamins plus B_T	25	0.93	17	29.8	24–33	12	70
Double vitamins plus B_T	26	0.95	24	29.5	24–33	20	83
No cholesterol	14	0.67	10	34.9	26–39	9	90
No thiamin	17	0.28	10	64.1	39–67	10	100
No riboflavin	19	0.39	6	42.1	30–47	6	100
No nicotinic acid	14	0.20	0	.....	.....	.....	0
No pyridoxin	16	0.38	10	45.6	42–47	9	90
No pantothenic acid	3	0.26	1	32.0	32	.....	0
No choline	14	0.13	2	79.0	79	1	50
No inositol	19	0.83	15	30.5	27–36	13	86
No biotin	19	0.76	18	32.2	28–35	15	83
No folic acid	25	0.98	18	31.2	28–38	13	72
No B_T	12	0.70	10	26.0	23–33	5	50

* Basic diet consists of casein 20 parts, glucose 80 parts, salts 2 parts, cholesterol 1 part except in no-cholesterol diet, and all vitamins but the one left out.

TABLE 3

GROWTH AND DEVELOPMENT OF *Tribolium* ON DIETS DEFICIENT IN INDIVIDUAL B VITAMINS

Addition to Basic Diet*	Larvae at 15 Days		Pupation		
	No.	Weight (Mg.)	No.	Av. Time (Days)	Spread (Days)
Yeast (5 per cent)	22	1.8	26	19.7	14–26
All vitamins	24	1.3	21	23.2	16–34
Double vitamins	29	1.3	27	22.8	18–32
No cholesterol	25	1.0	21	25.1	17–35
No thiamin	27	0.4	21	32.4	24–44
No riboflavin	18	0.6	10	32.1	24–42
No nicotinic acid	18	0.2	0	.....	.....
No pyridoxin	28	0.4	24	34.6	22–47
No pantothenic acid	1	0.2	0	.....	.....
No choline	27	1.5	24	21.8	16–36
No inositol	23	1.4	22	21.8	18–29
No biotin	21	1.2	18	27.3	22–38
No folic acid	17	1.4	16	23.6	18–37

* Basic diet consists of casein 50 parts, glucose 50 parts, salts 2 parts, cholesterol 1 part except in no-cholesterol test, and all vitamins but the one left out.

other closely related beetle and shares with *Palorus* the requirement for vitamin B_T. Lack of biotin and folic acid has a greater effect on this beetle than on *Palorus*, and lack of choline a lesser effect (Fraenkel *et al.*, 1950). Thus we see that three species of insects within the same tribe and family and occupying a more or less similar habitat have several important differences in their vitamin requirements. These differences can be extended to some of the other dietary components as well, both *Tenebrio* and *Palorus* requiring a much larger amount of carbohydrate than *Tribolium*. The latter is by far the most cosmopolitan of the three insects, exhibiting a wide range of adaptability to various diets and habitats.

TABLE 4

EFFECT OF THE ADDITION OF VARIOUS SUBSTANCES ON THE NUTRITIVE VALUE OF AN ARTIFICIAL DIET FOR *Palorus*

Addition to Basic Diet*	Larvae at 16 Days		Pupation		
	No.	Weight (Mg.)	No.	Av. Time (Days)	Spread (Days)
Yeast (5 per cent)	19	1.20	10	26.3	24–29
No addition	2	0.85	1	20.0	20
No addition (*Tribolium*)	16	1.70	14	25.0	23–29
B_T concentrate (48 μgm/gm of diet)	16	1.00	11	26.0	25–27
B_{12} (1 μgm/gm of diet)	3	1.00	2	22.5	20–25
Yeast extract (5 per cent)	18	1.05	14	26.4	24–30
Yeast (1 per cent)	17	1.15	12	25.6	24–27
Liver extract (1 per cent)†	20	0.94	7	27.4	26–30
Fat (corn oil) (1 per cent)	4	0.50	0	.....	.....
Control (wheat)	19	1.25	7	24.4	21–27

* Basic diet consists of casein, glucose, cholesterol, salts, water, and all vitamins.
† Nutritional Biochemicals Corporation.

B_T REQUIREMENTS

In an effort to explain the poor performance of the early artificial diets, a series of diets was prepared to test the effect of various accessory factors on *Palorus*. Table 4 indicates that, in addition to the eight B vitamins composing the all-vitamins mixture, B_T or a source of B_T, such as liver extract, yeast extract, or whey, was necessary for development.

B_T deficiency manifests itself in a high larval mortality, as may be seen in Table 4. It is also apparent that the few larvae that survive this lack of B_T develop and pupate normally. Thus the effect is in the quick death of the larvae rather than in their development. The effect of B_T deficiency is similar in *Tenebrio* larvae, which die in the early stages of their development. Table 4 also shows that corn oil and vitamin B_{12} are not needed by *Palorus*. The ability of *Tribolium* to develop normally without B_T is also shown.

Table 5 summarizes the results of an experiment in which the effects of graded doses of B_T were tested. When the quantities of the B_T concentrate were reduced, there was a heavy larval mortality below 0.75 μgm. of the concentrate per gram of the dry diet. This mortality occurred before the larvae were weighed at 17 days. However, when we consider the entire larval period as measured by the

number of larvae pupating, the minimum optimal dose is shown to be 1.5 or 3 μgm. of B_T concentrate per gram of the diet for *Palorus*. *Tenebrio* requires between 0.75 and 1.5 μgm. of the same concentrate per gram of the diet for good larval growth; no figures are available on the pupation requirements for that insect.

Since B_T was shown to be so critical for *Palorus*, storage of the vitamin in the larval tissue was investigated. Larvae were placed on artificial diets containing B_T, and groups of larvae were transferred every 3 days to B_T-deficient diets. Less than 9 days on the B_T diet resulted in a heavy larval mortality. The time to pupation remained fairly constant, but the number of larvae pupating declined after less than 12 days on the B_T diet. Good adult emergence from the pupae required 21 days on the B_T diet.

TABLE 5

EFFECT OF GRADED AMOUNTS OF A VITAMIN B_T CONCENTRATE ON THE GROWTH AND DEVELOPMENT OF *Palorus*

Addition to Basic Diet*	Larvae at 17 Days		Pupation			Adult Emergence	
	No.	Weight (Mg.)	No.	Av. Time (Days)	Spread (Days)	No.	Per Cent
No addition	10	0.40	2	39.0	38–40	2	100
B_T (0.19 μgm/gm)	12	0.52	4	33.2	32–35	1	25
B_T (0.37 μgm/gm)	13	0.60	4	32.0	25–35	2	50
B_T (0.75 μgm/gm)	32	0.84	8	33.4	32–36	4	50
B_T (1.5 μgm/gm)	39	0.96	14	31.5	28–38	9	62
B_T (3 μgm/gm)	39	1.01	24	31.7	27–34	21	87
B_T (6 μgm/gm)	34	0.93	16	31.9	28–40	10	62
B_T (12 μgm/gm)	37	0.97	19	30.6	27–35	16	84
Yeast (5 per cent)	39	1.19	10	27.1	24–31	6	60

* Basic diet includes casein, glucose, cholesterol, salts, water, and all vitamins.

CHOLINE REQUIREMENTS

The extreme susceptibility of *Palorus* to a lack of choline raised the question of the minimum optimal amount required. Consequently, experiments were conducted, using from 16 to 8,000 μgm. of choline per gram of the diet. These studies showed that every increase in the amount of choline from 16 to 1,000 μgm/gm resulted in a corresponding increase in the efficiency of the diet. Figure 1 shows the effect of added choline on larval development, illustrating the results of two experiments. Figure 2 shows the influence of added choline on the number of pupae and the rate of pupation. These figures show that the optimal amount of choline is 1,000 μgm/gm of diet, and this amount was routinely used thereafter in the all-vitamins mixture. The close correlation between the amount of choline in the diet and larval weight in *Palorus* suggests the use of this insect in bioassays for choline.

DISCUSSION

The basic food requirements of *Palorus* are similar to those of other insects. Insects, in general, need protein, carbohydrate, salts, a sterol, and most vitamins of the B complex (Trager, 1947; Fraenkel *et al.*, 1950). The essential B vitamins for *Palorus*, *Tenebrio*, and *Tribolium*—three closely related stored-products insects—are thiamin, riboflа-

vin, nicotinic acid, pyridoxin, and pantothenic acid. These insects, however, differ somewhat with respect to the other B vitamins. B_T and choline were necessary for *Palorus;* B_T was essential for *Tenebrio*, with choline, biotin, and folic acid also of importance (Fraenkel *et al.*, 1950); and in the present tests, biotin was found necessary for *Tribolium* but not

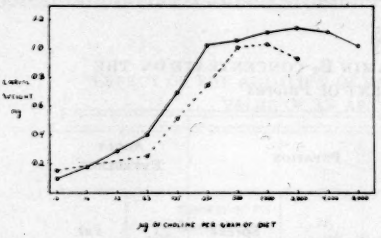

FIG. 1.—Effect of graded amounts of choline chloride on larval weight in *Palorus* after 17 days, showing results of two experiments. The basic diet used was that described on page 21.

for *Palorus*. "Vitamin-free" casein, however, has previously been shown to contain folic acid as an impurity (Fraenkel and Blewett, 1947), and the same seems to hold for biotin, judging from recent experiments. It is thus possible that *Palorus* might need folic acid and biotin on a basic diet of sufficient purity.

Little or no growth occurred in *Palorus* without added choline, 1,000 μgm/gm of the diet being optimal. Large quantities of choline are also required by the roach (Noland *et al.*, 1949), European corn borer (Beck *et al.*, 1949), black carpet beetle (Moore, 1946), and *Ptinus tectus* (Fraenkel and Blewett, 1943). *Tenebrio* responded to the absence of choline in some experiments but not in others (Fraenkel *et al.*, 1950). *Lasioderma serricorne* also required choline in one experiment (Fraenkel and Blewett, 1943) but not in a later experiment (Pant and Fraenkel, 1950). When the symbiotic yeasts were removed from *Lasioderma*, the larvae grew normally on an artificial diet supplemented with the all-vitamins mixture. When choline was omitted from the all-vitamins mixture, however, symbiont-free larvae failed to grow. This showed that the symbionts in *Lasioderma* supply this insect with choline. Intestinal synthesis of choline may explain the reason why some insects, such as *Tribolium* and *Silvanus surinamensis* (Fraenkel and Blewett, 1943), did not require a dietary source of choline. It is also possible that other compounds may replace the need for all or part of the choline in these insects, especially with reference to the need for labile methyl groups. In the chicken (McKittrick, 1947), the rat (Supplee *et al.*, 1945), and the baby pig (Neumann *et al.*, 1949), methionine and other methyl donors can replace all or part of the choline. This may explain the

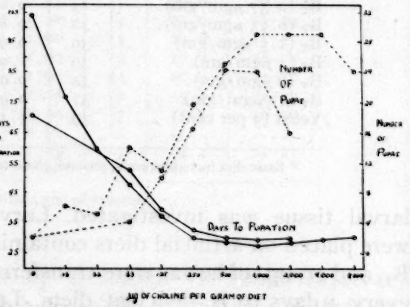

FIG. 2.—Effect of graded amounts of choline chloride on the average time to pupation and number of *Palorus* larvae pupating. Two experiments are shown. The basic diet used was that described on page 21.

results which Pant and Fraenkel (1950) obtained with *Stegobium paniceum*, where, in the absence of choline, this insect grew as well without its symbionts as with them.

Use of artificial diets for *Palorus* often resulted in heavy mortality, even when supplemented by the necessary vitamins or yeast. This also occurred, at times,

when the insects were on grain diets. Some mortality always occurred when rearing *Palorus* on test diets, even under optimal conditions. Slight changes in temperature and humidity might have been responsible for this mortality, but a more likely cause was the mechanical disturbance of the larvae when they were removed for weighing or for determining the pupation rate. Mature larvae hollow out cells in the food medium, where they remain for some time before pupating. These cells are broken when larvae are examined, and this must exert some physiological effects on the insects. Exposed larvae would, for example, be much more susceptible to desiccation than larvae within a cell. This destruction of cells is unavoidable and is a possible objection to the use of this insect for nutritional studies. However, larvae on control diets were also exposed, and thus the effect of these nonnutritive factors could be equalized.

SUMMARY

An artificial diet consisting of casein, glucose, cholesterol, salts, and a source of vitamins permitted satisfactory growth and development of the small-eyed flour beetle, *P. ratzeburgi* Wissman. *Palorus* required a sterol and a large quantity of carbohydrate, but only a small amount of protein. By the use of this artificial diet, the essential vitamins for this insect were found to be thiamin, riboflavin, nicotinic acid, pyridoxin, pantothenic acid, choline, and B_T. Biotin, folic acid, and inositol were apparently not required. Vitamin requirements differ somewhat among such closely related species as *Palorus*, *Tenebrio*, and *Tribolium*.

The effect of a B_T deficiency in *Palorus* closely resembles that in *Tenebrio*. A severe larval mortality occurs in the absence of B_T. Minimal dosages of a particular B_T concentrate for optimal larval survival were 0.75 μgm/gm for *Palorus*, and between 0.75 and 1.5 μgm/gm for *Tenebrio*.

When the amount of choline in the diet was increased up to 1,000 μgm/gm of the diet, there was a corresponding increase in larval growth and the number of pupae formed, and a decrease in the time required for pupation.

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THE EFFECTS OF VARIOUS CATIONS ON INSECT MUSCLE[1]

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SINCE Sidney Ringer's pioneering work, many physiologists have studied the effects of Na^+, K^+, Mg^{++}, and Ca^{++} on vertebrate muscle. Typically, when a vertebrate skeletal muscle, of a frog, for example, is immersed in an isotonic solution of a sodium salt, the muscle undergoes a rhythmic twitching. Apparently all the fibers in the muscle do not contract together, but individual fibers or groups of fibers contract independently, thus causing the muscle to quiver. The potassium ion may have two effects on invertebrate muscle; depending upon the concentration, it may cause (1) loss of irritability or (2) pronounced contracture (irreversible shortening). In frog and dog skeletal muscle the magnesium ion has been shown to have an anesthetic action. This anesthetic effect has been demonstrated both in frog muscles without nervous connections and in isolated frog muscle fibers (Ashkenaz, 1938). Vertebrate skeletal muscle protoplasm is very sensitive to the calcium ion. When a calcium salt is injected into a muscle fiber or if the cut end of a fiber is exposed to the calcium ion, there is an immediate and pronounced shortening of the fiber. Even an uninjured fiber shortens when exposed to calcium; however, this shortening requires a long period of time. For discussion of the above effects see Heilbrunn (1943).

With the exception of studies on heart muscle, relatively little work has been done on the effects of cations on invertebrate muscle. The experiments described in this paper were undertaken to determine whether, as a result of electrical stimulation, a response (shortening) occurs in invertebrate muscles following treatment with one or more of the four cations common to protoplasm.

MATERIAL AND METHODS

Studies were made on muscles of the honey bee (*Apis mellifica*) and the cockroach (*Periplaneta americana*). Both of these species were collected in the Philadelphia area.

In the case of the honey bee (*A. mellifica*), only the indirect wing muscles (tergo-sternal) were used. These muscles (see Snodgrass, 1925) were removed from the thorax, and single fibers were isolated in Bělař's insect Ringer by cutting the fibers, as close as possible to the attached chitinous thorax wall, with fine steel needles. The single fibers were transferred with a pipette, along with insect Ringer, to a depression slide. Rather than transfer the fibers to other slides containing test solutions, it was much

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simpler to pipette off the Ringer and replace it with test solutions (Ashkenaz, 1938). However, because of the difficulties involved in stimulating wing muscle fibers of the honey bee, most of the experiments reported in this paper are concerned with the effects of cations on cockroach muscle.

Cockroaches were immersed in water (Yeager and Hager, 1934) until they became inactive. The femur was cut just above the tibio-femoral joint and just below the coxal-femoral joint. After splitting the chitin of the femur longitudinally, the extensor muscle was located and easily removed by grasping its tendon with forceps. The entire muscle was transferred immediately to a slide containing "cockroach Ringer." These muscles were washed three times in the solution to be tested. These washings were completed in less than 40 seconds.

The apparatus used for electrically stimulating the insect muscles consisted essentially of an inductorium, two 1.5-volt dry cells, and a pair of platinum electrodes. One electrode was placed at each end of the muscle, or muscle fiber, in such a manner as to be as close as possible to, but not in contact with, the muscle. Whole muscles of the cockroach were observed under a Spencer dissecting microscope with a magnification of 20.7×. The criterion of response was the visible movement (shortening) of the muscle upon stimulation with a single "make" shock. In the case of the bee muscle fibers, both single and repetitive shocks were used as stimuli. During stimulation these muscle fibers were observed with a compound microscope with a magnification of 440×.

Merck's reagent grade chemicals and glass (Pyrex) doubly distilled water were used exclusively in the preparation of all solutions. Bělař's insect Ringer (Bělař, 1929), with calcium chloride omitted, was used as the dissecting medium for the bee muscle. This calcium-free insect Ringer had the following composition: 20 cc. of 9 per cent NaCl; 4 cc. of 1 per cent KCl; 0.4 cc. of 10 per cent $NaHCO_3$; 5–10 cc. of 10 per cent dextrose; and distilled water to make 200 cc. of solution. In all experiments involving the study of cockroach muscles, a fluid was used having the same composition as the one described by Yeager and Hager (1934) in their work on heart muscle of the cockroach (*P. orientalis*). This fluid, which in the present paper will be termed "cockroach Ringer fluid," contained 9.82 gm. NaCl, 0.77 gm. KCl, 0.50 gm. $CaCl_2$, 0.18 gm. $NaHCO_3$, 0.01 gm. NaH_2PO_4, and 1.0 gm. glucose in a liter of solution. The pH of both solutions was maintained at values from 6.9 to 8.6.

Stock solutions of pure chloride salts of the four cations were prepared, and these solutions were diluted with calcium-free Ringer to the desired concentration of the cation to be studied.

EXPERIMENTAL RESULTS

Wing muscles of the bee usually responded to electrical stimulation only if they were immersed in Bělař's Ringer while still intact in the thorax. No single fibers or groups of fibers were ever seen to respond to electrical stimulation (single shock or tetanic shock from an inductorium connected to from two to twelve dry cells) while immersed either in various salt solutions or in modifications of Bělař's Ringer fluid, even though the times required for dissection were short (5–10 minutes). It is true that prolonged tetanic shocks (5–10 seconds) caused these fibers to shorten. However, this shortening seemed to be due to temperature effects, since there was evidence of excessive heat during these tetanic stimulations. This heat was apparently pro-

duced by the high electrical resistance of the fibers.

Extensor muscles of the cockroach, when still attached to the thorax, remained irritable for as long as 6 hours. Even when separated from the thorax, these muscles remained irritable for as long as 2 hours after their isolation. In one case attached leg muscles were found to be irritable after immersion in insect Ringer fluid for a period of 23 hours.

TABLE 1

EFFECTS OF POTASSIUM CHLORIDE
Cockroach (*P. americana*)

No. of Muscles	Concentration of KCl	Time in KCl (Min.)	Response to KCl		Time for Loss of Irritability (Sec.)
			Contraction Period (Sec.)	Duration of Quivering (Sec.)	
3.....	0.58 M*	1.0	2	30	30
2.....	0.39 M*	2.0	2	27.5	90
3.....	0.25 M	1.25	2	.....	55
3.....	0.23 M	1.25	2	20	60
3.....	0.21 M	1.66	2	.....	95
9.....	0.19 M	1.5	2	25	90
9.....	0.19 M*	1.5	2	18.5	60
4.....	0.16 M	1.5	2	15	35
3.....	0.15 M	1.75	2	30	100†
4.....	0.12 M	1.33	2	25	65
6.....	0.09 M*	1.33	2	10	60‡

* Pure solutions of KCl.
† Two muscles.
‡ Four muscles.

As may be seen from the results presented in Table 1, solutions of KCl produce immediate contracture (irreversible shortening) of leg muscles of the cockroach and subsequently cause a loss of irritability. Test solutions were prepared by adding 0.392 M KCl to definite portions of insect Ringer fluid. Contracture occurred in 2 seconds in all solutions of KCl tested. Following this shortening, the muscles usually showed a pronounced quivering, which lasted from 10 to 30 seconds. After exposure to hypertonic solutions of KCl (above 0.19 M), muscles did not recover their irritability in insect Ringer fluid; but, after exposure to hypotonic KCl solutions (below 0.19 M), nearly half the muscles observed regained their irritability in insect Ringer fluid. In no case did muscles recover after exposure to any solution of KCl for 1.5 minutes or longer.

Solutions of $MgCl_2$ have a very pronounced effect on the irritability of the leg muscles of cockroach. Magnesium caused a definite anesthesia of this muscle (see Table 2). This anesthetic effect occurred in less than 1.5 minutes in hypertonic (0.2–0.28 M) solutions of $MgCl_2$, in 2–4 minutes in isosmotic (0.14 M solutions), and in less than 5 minutes in hypotonic (0.1–0.07 M) solutions. All these muscles recovered their irritability within 1 minute after being returned to insect Ringer fluid.

Sodium chloride solutions have little or no effect on the irritability of cockroach leg muscles except after 18 minutes' exposure. Muscles immersed in NaCl solutions showed normal irritability for 15 minutes or more (see Table 3). After these relatively long periods of immersion, muscles gradually lost their irritability. Those muscles which lost most of their irritability after long exposure to NaCl could recover almost normal irritability in insect Ringer fluid.

The irritability of cockroach leg muscles was not changed appreciably after immersion in isosmotic (0.14 M) $CaCl_2$ for 5 minutes. However, after 5 minutes' immersion in isosmotic $CaCl_2$ there was a gradual decrease in irritability. But some of the muscles retained their irritability even after 15 minutes' immersion in $CaCl_2$ (see Table 4). Once a muscle had ceased to respond to electrical stimulation in $CaCl_2$, it did not recover its irritability in insect Ringer fluid.

Six muscles, which had become anes-

thetized previously by magnesium (3.5 minutes' immersion in 0.14 M $MgCl_2$), recovered their irritability in 1 minute after immersion in isosmotic (0.14 M) $CaCl_2$, whereas three muscles, previously anesthetized by exposure to 0.14 M $MgCl_2$ for 4 minutes, recovered their irritability in 5 minutes after being returned to 0.19 M NaCl (bottom of Table 2).

Muscles which have lost their irritability by exposure to 12.5 per cent sucrose and 7 per cent dextrose do not regain this irritability when transferred to isosmotic (0.19 M) NaCl. However, following exposure to sucrose, insect Ringer fluid restores the irritability of whole muscles of the cockroach in less than 2 minutes. Likewise after loss of irritability in 12.5 per cent sucrose (5 minutes' exposure), isosmotic (0.14 M) $CaCl_2$ produced recovery in 2 minutes (Table 5).

TABLE 2

MAGNESIUM ANESTHESIA AND RECOVERY IN VARIOUS SALT SOLUTIONS
Cockroach (*P. americana*)

No. of Muscles	Concentration of $MgCl_2$	Time in $MgCl_2$ (Min.)	Time Required for Anesthesia in $MgCl_2$ (Min.)	Recovery Salt Solution	Time in Salt Solution (Min.)	Time Required for Recovery in Salt Solution (Min.)
4.....	0.28 M	1.66	1.25	Ringer	1.5	1.0
3.....	0.2 M	1.75	1.5	Ringer	1.33	1.0
7.....	0.14 M	2.33	2.0	Ringer	1.5	1.0
4.....	0.1 M	3.5	3.0	Ringer	1.3	1.0
4.....	0.07 M	5.66	5.0	Ringer	1.6	1.0
6.....	0.14 M	4.5	3.5	0.14 M $CaCl_2$	4.0	1.0
3.....	0.14 M	4.5	4.0	0.19 M NaCl	5.33	5.0

TABLE 3

IRRITABILITY IN SODIUM CHLORIDE SOLUTIONS
Cockroach (*P. americana*)

No. of Muscles	Concentration of NaCl	Time in NaCl Solutions (Min.)	Response to Single Shock in NaCl
4.....	0.25 M	16.0	+
4.....	0.21 M	16.0	+
5.....	0.19 M	15.0	+
2.....	0.17 M	6.0	+
3.....	0.15 M	18.0	+
2.....	0.10 M	9.0	+

DISCUSSION

There is some controversy as to whether isolated wing muscles in insects do respond to electrical stimulation. The results of Liang (1936) with honey bee and Cremer (1934) and Heidermanns (1931) with dragonflies show that wing muscles of these forms do contract under the influence of electrical stimulation. On the other hand, recent investigations of Pringle (1949) and Roeder (1951) reveal that attempts to stimulate with an electric current isolated or partly isolated indirect flight muscles of flies are unsuccessful.

Pringle states that no gross damage is done to the musculature of the thorax if a fly is "bisected vertically" with a sharp instrument. That there is little upset to the nervous system is shown by the fact that the portion containing the thoracic ganglion shows normal leg reflexes (Pringle, 1940). In experiments described in this paper the honey bee wing

muscles were usually sensitive to electrical stimulation only while intact in the animal. However, in no case was any response to electrical stimulation evidenced after partial or complete dissection, even though the dissection periods were of short duration. This would indicate that, in the honey bee at least, there is some disturbance incident to dissection. A critical discussion of the mechanism involved in the loss of irritability of the dissected fiber is not pertinent at this time. However, Pringle (1949) has suggested that the frequency of contraction of the wing muscles is not determined centrally in the ganglion but is controlled directly by the loading upon them. A study of the anatomy of these muscles reveals that there are two sets of muscles which act antagonistically in their operation of the wings. Bisection of the thorax upsets the loading on the muscles by alteration of the antagonism between the longitudinal bundles. Although the muscles may be excited by electrical stimulation, no contraction occurs (for discussion see Pringle, 1949).

TABLE 4

IRRITABILITY IN CALCIUM CHLORIDE
Cockroach (*P. americana*)

No. of Muscles	Concentration of $CaCl_2$	Time in $CaCl_2$ (Min.)	Response to Single Shock in $CaCl_2$*	Response to Single Shock in Ringer*
10.....	0.4 M	1.6	−	−
10.....	0.378 M†	2.0	−	−
2.....	0.348 M†	3.0	−	−
4.....	0.315 M†	3.0	−	−
4.....	0.21 M	3.0	+	+
3.....	0.18 M	3.0	+	+
3.....	0.168 M†	3.0	+	+
14.....	0.14 M	5.3	+	+
6.....	0.14 M	15.0	+	+‡ −§
2.....	0.13 M	5.0	+	+
2.....	0.11 M	3.0	+	+
2.....	0.09 M	3.5	+	+
5.....	0.07 M	3.0	+	+
2.....	0.035 M†	4.7	+	+

* Plus sign (+) indicates response; minus sign (−) indicates no response.
† Mixed solutions of $CaCl_2$ and Ringer.
‡ Two muscles.
§ Four muscles.

Because of the difficulties encountered in attempts to stimulate wing muscles of the honey bee electrically, preparations of cockroach leg muscles were found preferable. Whole leg muscles of the cockroach responded readily to electrical stimulation. Thus a viable preparation of invertebrate muscle was available, suitable for testing the effects of the four major cations (sodium, potassium, calcium, and magnesium).

TABLE 5

RECOVERY OF IRRITABILITY (LOST IN SUGAR SOLUTIONS) IN SALINE SOLUTIONS
Cockroach (*P. americana*)

No. of Muscles	Concentration of Sugar Solution	Time in Sugar Solution (Min.)	Response in Sugar Solution (Single Shock)	Concentration of Saline Solution	Time in Saline Solution (Min.)	Response in Saline Solution (Single Shock)
6.....	12.5% sucrose	4.66	No response after 4 min.	Ringer	2.0	Response after 1 min.
8.....	12.5% sucrose	6.66	No response after 5 min.	0.14 M $CaCl_2$	2.66	Response after 2 min.
9.....	12.5% sucrose	7.0	No response after 5 min.	0.19 M NaCl	3.55	No response after 3.3 min.
5.....	7.0% dextrose	8.33	No response after 8 min.	0.19 M NaCl	4.0	No response after 3 min.

Potassium chloride has two obvious effects on whole muscles of the leg of the cockroach (*P. americana*). There is first a contraction (contracture) of the muscle,

which occurs almost immediately in hypotonic, isotonic, and hypertonic solutions of potassium chloride. This is followed by a gradual loss of irritability of the muscle exposed to the potassium chloride solution. This loss of irritability due to the effects of isotonic and hypotonic solutions of potassium chloride could be regained upon return of the muscles to normal cockroach Ringer fluid.

The anesthetic effects of magnesium are well known. These effects are not confined to muscle alone but have been reported in various marine eggs (Heilbrunn, 1934), nerves, and other tissues. Magnesium likewise has an anesthetic effect on muscles of the cockroach. It is believed that this anesthetic effect can be antagonized by the presence of the calcium ion (Ashkenaz, 1938).

Sodium chloride solutions have little or no effect on the irritability of cockroach leg muscle. It has been stated by Overton (1902) that, once frog muscles have lost their irritability in sugar solutions, this irritability could be regained when the muscle was transferred to a solution of sodium salt. Cockroach muscles, after immersion in sucrose or dextrose solutions, did not regain their irritability when transferred to sodium chloride solutions. However, the irritability lost in sucrose could be regained by immersion in normal Ringer solution (containing calcium) and in stronger calcium solutions. This evidence supports the findings of Heilbrunn and Ashkenaz (1941) on vertebrate (frog) muscle.

As far as the irritability of the muscle is concerned, slightly hypertonic (0.21 M) and solutions of calcium chloride weaker than 0.21 M had no apparent effect on the irritability of the muscles after relatively long periods of exposure, 3 minutes or more. Shortening of muscle was observed when the muscles were immersed in the calcium solution, but no quantitative measurements were made.

In view of the evidence presented, it may be concluded that the insect muscles used in these experiments follow the same pattern in their response to the four major cations as do vertebrate (frog) muscles. (The question of the irritability of the honey bee wing muscles is still in doubt.) The effects of these cations on muscles of the cockroach are in agreement with the earlier work on vertebrate muscle.

SUMMARY

1. The question of the irritability of wing muscles in insects is briefly discussed.

2. Solutions of potassium chloride have two effects on leg muscles of the cockroach. First, there is a rapid contracture. This is followed by a gradual loss of irritability. Irritability lost in hypotonic and isotonic solutions of potassium chloride could be regained upon return to normal cockroach Ringer.

3. Magnesium has an anesthetic effect on cockroach muscles. This anesthesia is neutralized by exposure of the muscles to normal cockroach Ringer and to solutions of calcium chloride.

4. Sodium chloride has no effect on the irritability of cockroach muscle. Once the irritability of these muscles has been lost by immersion in sugar solution, isotonic NaCl is unable to restore this irritability.

5. Slightly hypertonic solutions of calcium chloride have little or no effect on the irritability of cockroach muscle.

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COMPARATIVE ECOLOGY AND PHYSIOLOGY OF THE CRAYFISH ORCONECTES PROPINQUUS AND CAMBARUS FODIENS[1]

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IN THE early years of the present century Ortmann (1905, 1906, 1913) assembled and interpreted the information concerning crayfish distribution. Although these contributions were largely of a taxonomic and geographic nature, they nevertheless have considerable ecological content. Since Ortmann's publications, the writings in this field have been primarily either state surveys or specific taxonomic or physiological studies. The present investigation is an attempt to relate certain ecological and physiological differences and particularly to analyze environmental action on the population.

In the Chicago area, where this work was done, several crayfish species occupy a wide ecological range of freshwater habitats. Two species in particular tend to be restricted, one to the lotic extreme of clear, rock-bottomed, flowing waters and the other to the lentic extreme of stagnant, mud-bottomed, often temporary ponds. *Orconectes propinquus propinquus* Girard (= *Cambarus propinquus propinquus*) occupies the clear streams, and *Cambarus fodiens* Cottle (= *Cambarus argillicola*) is the characteristic woodland-pond form.

The local distribution was determined by systematic collecting, and the data were assembled into an ecological catalogue. A relatively small area was chosen for intensive study of the habits and populations of *O. propinquus* and *C. fodiens*. Part I of this paper will report these field data.

The association between the factors of these diverse environments and their crayfish populations suggested general causal relations. But, for a more complete un-

[1] The author wishes to express his gratitude to Dr. Thomas Park for initiating and directing this investigation and for his encouragement throughout. He is also indebted to Drs. W. C. Allee and Sewall Wright for their criticism and helpful suggestions. Help from Dr. H. H. Hobbs, Jr., University of Virginia, in identification of specimens is gratefully acknowledged.

derstanding, it was necessary to analyze more specifically the physiological reaction to each pertinent environmental factor. Part II is concerned with these investigations; the several ecological differences of the lotic and lentic habitats are related to the crayfish populations by means of experimental toleration physiology and behavior studies. Part III evaluates the field and laboratory data as demonstrating contemporary environmental action.

I. Field Studies

REGIONAL SURVEY

PHYSIOGRAPHY

The area surveyed forms a segment of a discrete physiographic unit; it is a portion of the continuous belt of terminal moraine about the base of Lake Michigan. This ridge of small hills rises abruptly from the plains of Chicago to the northeast and merges into the Illinois plains to the southwest. North of the point of study the moraine is bisected by the Sag Canal. Prior to alterations, this belt of terminal moraine was part of the major divide between the Mississippi and the St. Lawrence drainage systems.

There are no large natural lakes in these uplands, but numerous small ponds occupy depressions among the hills. These are all muck-bottomed and relatively shallow. The woodland ponds are characteristically vernal, being present as bodies of water only in the spring and drying in the summer to low meadow. The classical successional sere may be observed, from deeper ponds, drying only in very dry summers, to those which are filled only during spring and autumn rains.

Postglacial stream dissection has reached into both sides of the moraine, from the Sag and Little Calumet to the east and from the Des Plaines drainage to the west. Upper reaches of these streams are unpolluted. The flowing waters tend to be of three types: (1) intermittently flowing headwaters and oxbows; (2) swift, clear, gravel- or rock-bottomed streams; and (3) slowly moving, mud-bottomed streams. Since the sources of some of these streams are in relatively level uplands, the linear sequence may be: slowly moving, mud-bottomed, and intermittent headwaters, followed by downcutting, rapid stretches, and, at the mouth, a wider, slower, mud-bottomed segment.

REGIONAL CRAYFISH[2]

Seven species of crayfish were identified from the collections taken from the lengths of ten stream systems and ten ponds. The standing-water fauna was dominated by *C. fodiens* (Cottle) and *Procambarus blandingii acutus* (Girard); present in smaller numbers were *C. diogenes* (Girard), *C. gracilis* (Bundy), and *O. immunis* × *pedianus* (Hagen). The intermittently flowing streams were generally devoid of crayfish, but, where connected directly to ponds, they contained the pond fauna; this was true of the upper reachers of Hickory Creek. The slowly moving stream fauna characteristically included *P. blandingii acutus* (Girard) and in some instances *C. diogenes* (Girard) and *O. virilis* (Hagen). The rapidly flowing streams contained *O. propinquus* (Girard) and *O. virilis* (Hagen) as dominant species, with *P. blandingii acutus* (Girard) and *C. diogenes* (Girard) in smaller numbers (see Table 1).

The diversity of habitat is immediately apparent. Equally apparent is the restriction of two species to environmental extremes: *O. propinquus* to swiftly flowing streams and *C. fodiens* to ponds.

[2] Generic and specific names, here and later, are those of Hobbs (1942).

This distributional pattern has been noted by the many investigators who have recorded these species. This relationship of species to habitat is the essential core of this investigation. The two forms are particularly suited for a comparative ecological study. They comprise the largest species population of their respective habitats, may be collected in numbers, and are very similar in body size. Suitable sites for specific study are found in closely adjacent areas.

TABLE 1

AN ECOLOGICAL CATALOGUE OF THE CRAYFISH OF THE CHICAGO AREA

I. Streams
- Swift, rock-bottomed streams
  - *Orconectes propinquus*
  - *Orconectes virilis*
  - *Procambarus blandingii acutus*
  - *Cambarus diogenes*
- Slow, mud-bottomed streams
  - *Procambarus blandingii acutus*
  - *Orconectes virilis*
  - *Cambarus diogenes*

II. Ponds
- Temporary woodland and open ponds
  - *Cambarus fodiens*
  - *Procambarus blandingii acutus*
  - *Cambarus diogenes*
- Permanent woodland and open ponds
  - *Cambarus fodiens*
  - *Procambarus blandingii acutus*
  - *Cambarus diogenes*
  - *Orconectes immunis*
  - *Cambarus gracilis*

DESCRIPTION OF SPECIFIC STUDY SITES

An area containing ponds and streams within $\frac{1}{2}$ square mile was selected for intensive study. The sites are included in the Tinley Grove Forest Preserve in the Palos Park region at Harlem Avenue and 143d Street south. This particular tract is part of the extensive park system of Cook County, Illinois. The specific sites were Tinley Creek, east of Harlem Avenue, and Tinley Pond I (see Fig. 1).

This designated area is part of the Tinley terminal moraine, the innermost of the series. It arises abruptly from the Chicago plains (old Lake Chicago bottom) and merges into the large Valparaiso moraine to the west and south. The hills are gently sloped, and the elevations range from 630 to 670 feet. While the region is physiographically young, considerable dissection has occurred at the eastern margin of the moraine, and the streams have reached up into the Valparaiso levels. Drainage at this point is eastward.

The pond and stream are closely contiguous, and they have, therefore, many environmental similarities. They are both affected equally by meteorological conditions. A common basic substrate is composed of glacial till, largely of clay and rock fragments; underlying is bedrock of Niagara limestone glacially scoured (Bretz, 1939). The land vegetation of both habitats is largely second-growth mixed hardwoods.

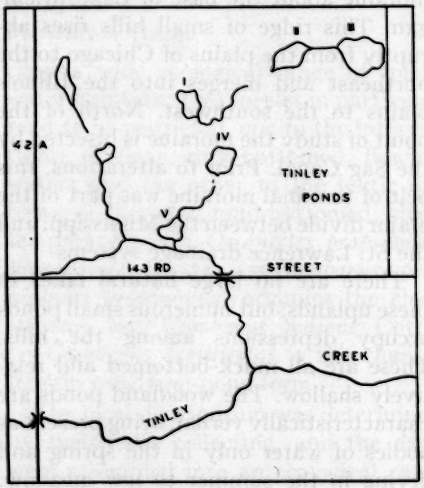

FIG. 1.—Specific study site, a portion of the Tinley Grove Forest Preserve at Harlem Avenue (42A) and 143d Street, Cook County, Illinois.

The different, rather than common,

factors are probably critical in differential action on the crayfish faunae. It was on these differences that field analysis was focused.

TINLEY CREEK

Tinley Creek flows northeasterly and is approximately 4 miles in length. The uppermost mile is intermittently flowing. The stream course is bordered by gallery forest and pasture lands; at no point is it directly polluted. The mouth of the creek is confluent with the Sag Canal east of U.S. Highway 50. The valley at the study site is young, but some small plains and oxbows have formed. The bed is cut through clay and the contained rock fragments; the limestone bedrock is reached in the lower stretches. The gradient for this ½ mile is 5 feet per thousand; the drop occurs in riffle stretches. Depth and current vary with seasonal changes in water table and runoff. Normal width is from 6 to 14 feet. No permanent flora is found; at times of low water, algal blooms may develop but are ephemeral.

TINLEY PONDS

As previously mentioned, the ponds studied fall into the classical successional types. Several ponds are located in the area; Pond I was selected as a typical intermediate type; it is water-filled from November to July. Soil borings reveal an underlying clay covered with organic muck, ranging in depth from a few inches marginally to several feet in the center of the pond. When filled, the pond has only a small area of open water, the greater part of the surface being covered with several sedges and grasses; the common cattail (*Typha latifolia*) occupies most of the pond. With summer drying, the pond floor is covered with meadow grasses. Seepage between ponds is considerable in the spring months, and in some cases they are actually drained by runoff brooks. The largest of these vernal streams is a small, unnamed brook which will be called "Tinley Brook" (see Fig. 1). It connects one of the ponds directly and others secondarily with Tinley Creek. The brook is narrow, physiographically very young, and has a gradient over its entire length of 10 feet per thousand. The volume of water and rate of flow is never large, even in spring flood.

These two study sites were visited regularly over a period of 3 years, and recordings were made of specific environmental factors for the calendar year 1948. These will be discussed as comparisons between the two habitats.

ANNUAL VARIATIONS IN ENVIRONMENTAL FACTORS

Two basic differences between pond and stream are apparent—water movement and the specific difference locally in permanent versus temporary water. Reflecting these basic differences are certain other physical and chemical factors.

Recordings were made of water depth and movement, temperature, and calcium carbonate and dissolved oxygen content. Samples and measurements were taken at consistent stations in the center of a stream-riffles area and in the open water of Pond I.

CHANGES IN WATER DEPTH

Tinley Creek at the study station never ceased flowing at any time of the year but rose and fell directly with the ground-water table. Winter depth was constant at approximately 1 foot. With spring thaw and rain, flood peaks reached over 2 feet in the rapids. Water depth declined gradually through the spring, with occasional peaks following heavy rains. By early summer the level had fallen to a few inches, with bottom rocks exposed.

This low level persisted throughout the summer and fall until heavy rains brought the depth to the late fall and winter levels.

The pond differs radically in being summer-dry. Like the stream, its water depth was greatest during the spring thaw and rains. Since the basin is shallow, the pond has a maximum depth of near 17 inches; when filled to this level in the spring, runoff occurs. The decline through May and June was halted by heavy rains, but the drop in depth followed the declining water table, and complete drying occurred by July. The final stages were rapid, from a water level of a few inches above, to 2 feet below, ground in a week's time. The bottom was green with grasses within a week of final drying. Varying with the season, the summer water table may be a few to several feet below ground. The pond basin remained dry until fall rains raised the water table to the pond level. The fall period of open water was short, the pond freezing solidly in the winter months (see Fig. 2).

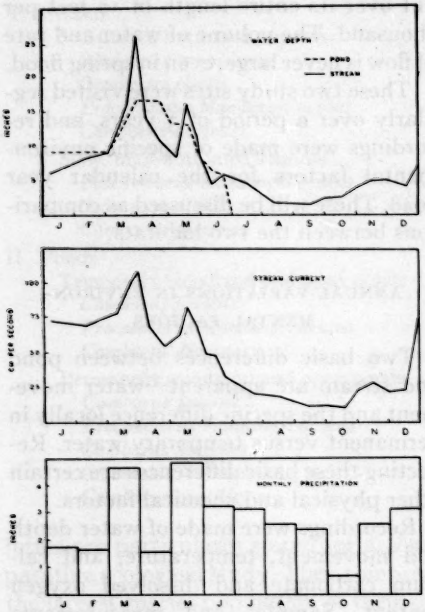
WATER DEPTH
POND
STREAM
INCHES
J F M A M J J A S O N D
STREAM CURRENT
CM PER SECOND
J F M A M J J A S O N D
MONTHLY PRECIPITATION
INCHES
J F M A M J J A S O N D

FIG. 2.—Water depth of pond and stream (*upper*), stream current (*center*), and monthly precipitation (*lower*) for the year 1948. (Precipitation data from Dunn, 1948.)

VARIATIONS IN WATER MOVEMENT

Water movement as current is present only in the stream and is maintained throughout the year. Measurements were made in the rapid-water station by timing a weighed float over a short course. Records are reported in centimeters per second and, as the methods are very approximate, can be considered as of only comparative value.

Rate of flow followed the annual pattern of stream depth. Winter current tended to be constant and near 75 cm/sec. Concomitant with thaw and spring rains, the flood peak of over 1 meter per second was reached. Recurrent heavy rains caused sharp peaks in the generally declining rate of flow through the spring months. By June the current was reduced to 25 cm/sec and to less than this figure throughout the summer. An increase in flow followed fall rains.

Aside from surface wind ruffling, no water movement could be measured in the pond.

VARIATIONS IN TEMPERATURE

Water temperatures were recorded in degrees centigrade from mid-depth. In the stages prior to pond drying, top and bottom recordings were made.

The stream temperatures through the months of January and February remained consistently between 0° and 1°. Even the riffle stretches were ice-covered at this time. As air temperatures rose steadily through spring, the temperature of the water followed, but with considerable lag. By June and July, afternoon water temperatures were as high as 20°,

with air temperature at 30°. No recordings over 20° were made at any time during the summer months. With the fall drop in air temperatures, the water values declined until air temperatures fell below zero (Fig. 3).

The water of the pond is in a solid ice block through the winter months. With spring thaw, this block melts first at the

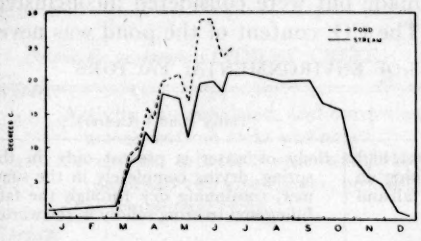

FIG. 3.—Water temperatures of pond and stream for the year 1948.

surface and progressively, with intermittent surface ice, down to the pond bottom. With release from ice, water temperature followed that of the air. By late spring, afternoon temperatures were equal to air recordings and therefore rose to near 30° by summer. Some stratification was observed until shortly before pond drying, at which time the pond became uniformly tepid by late afternoon. The pond margins warmed more rapidly than the center. After the drying of the pond, ground water remained consistently near 20°, irrespective of air or soil surface temperatures. After the late fall refilling of the pond, water temperatures dropped to near zero and remained so under the ice cover until complete freezing.

VARIATIONS IN DISSOLVED OXYGEN CONTENT

Water samples for determination of dissolved oxygen concentration were taken from mid-water. The analytical method used was the modified Winkler (Amer. Pub. Health Assoc., 1925). The mean of four samples was recorded.

The highest stream recording was made in the winter months before ice coverage. As high as 11 cc/liter were noted for December; under ice this value dropped to near 5 and then rose to 10 cc/liter after the ice broke up. With temperature increasing and agitation decreasing through the spring, the oxygen content dropped to a consistent summer level of near 7 cc/liter. A steady increase was noted through the fall to the winter levels. Fluctuations tended to be the rule, but at no time was the level far below saturation for the given temperature (see Fig. 4).

The oxygen content of the pond was low at two times of the year. As the pond froze in the early winter, the increasingly diminished body of water suffered a marked decline in oxygen concentration. With 10 inches of ice over 4 inches of water, the content was reduced to zero. Following the spring thaw, the oxygen

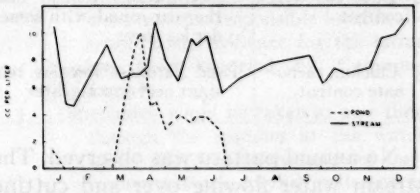

FIG. 4.—Dissolved oxygen content of pond and stream for the year 1948.

content rose to saturation. From an April high of approximately 8 cc/liter, the oxygen content dropped to between 3 and 4 cc/liter for the remainder of the spring. With the final rapid drop in water level, the content declined to 0.4 cc/liter, 1 week before drying. At times of intense algal bloom during this last phase, the content was found to rise during the day as a probable result of photosynthetic activity. Ground water below the pond

basin during its dry period contained between 2 and 3 cc/liter of dissolved oxygen.

VARIATIONS OF CALCIUM CARBONATE CONTENT

Neither Tinley Creek nor Tinley Pond I varied through the year in the total hardness of its water. The soap method of analysis was used, recorded in milligrams per liter of calcium carbonate (Amer. Pub. Health Assoc., 1923).

SUMMARY OF ANNUAL VARIATIONS OF ENVIRONMENTAL FACTORS

Water	Tinley Creek Riffles	Tinley Pond I (Center)
1 Depth	Body of water is constantly present, high during spring floods, diminishing in summer and increasing through fall and winter	Body of water is present only in the spring, drying completely in the summer, continuing dry through the fall, filling and freezing solidly in the winter
2 Rate of current	Water flow never ceases, but rate of current varies from flood heights in the spring to slow through the summer, increasing in the fall to winter rates	Water movement as current is completely absent
3 Temperature	Temperature through the winter remains near 0° C., rising with air temperatures to a maximum of approximately 20° through the summer and dropping to near zero with fall frost	Spring temperature lags behind the air but approaches air temperature in early summer prior to drying, with recordings up to 30°
4 Dissolved oxygen content	Concentrations remain near saturation the year round, with some decrease under ice cover	Spring concentrations starting at saturation diminish to less than 1 cc/liter just prior to pond drying
5 Calcium carbonate content	Total hardness remains relatively constant near 370 mg/liter	Total hardness remains relatively constant near 75 mg/liter

No annual pattern was observed. The stream water flowing over and cutting into clays and limestone was very hard, between 360 and 400 mg/liter. In the pond the original clay substrate has been covered with an extensive bed of organic muck. The water, while continuous with ground water in clay, may be derived largely from runoff surface water. The calcium carbonate content was one-quarter that of the stream, between 60 and 90 mg/liter.

ADDITIONAL FACTORS NOT INVESTIGATED

These previously described physical and chemical aspects of the two environments are a fraction of the total complex. They do represent the most obvious differences which might be predicted to be acting on the biota.

Certainly, many changes occur in the chemical milieu of stagnating pond waters. These would be lacking in the stream and could be toxic. Measurements of carbon dioxide and pH were made but were considered inconclusive. The CO_2 content of the pond was never greater than 3 mg/liter during final drying. This may probably be related to the reduced depth per surface area. The pH recordings were variable, and interpretation is difficult because of the variety of factors affecting the value (see accompanying tabulation).

LIFE-HISTORIES OF *O. propinquus* AND *C. fodiens*

Observations on the crayfish of Tinley Creek and Tinley Ponds were made over four breeding seasons. The data on *O. propinquus* corroborate the comprehensive work on the same species for central Illinois done by Van Deventer (1937).

Life-histories are lacking for *C. fodiens*, though many investigators have contributed to a general understanding.

The primary aim of this paper is not the detailed presention of the natural history of the two species of crayfish; therefore, an effort is made to condense the following information to an outline of those aspects pertinent to the ecological problem.

Orconectes propinquus (TINLEY CREEK)

Winter:

1. Activity was restricted, and burrowing among bottom rocks was noted
2. Specimens collected were adult but comprised two size classes; probably of two annual crops

Spring:

1. Activity increased with spring thaw as winter quiescence ended
2. Large numbers were collected in the strongest spring flood currents
3. Females became gravid in early May; at this time they remained in sheltered niches along stream margins between rocks and tree roots
4. Young appeared on the females late in May and early June
5. The early instars became free-swimming in June

Summer:

1. The young grew rapidly and underwent many molts
2. The number of young relative to adults increased
3. The yearling crop was mature by fall, but the individuals were smaller than those of the previous year's crop

Fall:

1. There is evidence that copulation occurs (Van Deventer, 1937)
2. Prior to freezing, activity diminished, and the numbers collected dropped markedly

Cambarus fodiens (TINLEY POND I)

Winter:

1. Since the pond is a solid block of ice, it seems certain that the crayfish population is confined below ground

Spring:

1. In March a 2-week time lag was noted between complete ice melting and first appearance of adult crayfish
2. Gravid females and some young also appeared at this time
3. Through the spring the young underwent many molts and comprised the largest part of the population
4. High adult mortality was noted throughout the spring
5. In June, shortly before summer drying, the young comprise the entire population of the open pond and are approximately half adult size; adults had probably burrowed; some marginal chimneys were noted
6. By 2 weeks prior to pond drying, only an occasional specimen was found; simultaneously, the pond margin became dotted with chimneys, which were built around openings of burrows that contained *C. fodiens*

Summer:

1. Water remaining in the deeper portions of the pond in its final drying stages became the burying ground for a large concentration of aquatic animals; among these were large numbers of salamander and frog larvae, snails, aquatic insects, isopods, and amphipods; no crayfish were found in this assemblage; burrowing and emigrations must be responsible for this absence; some evidence for the latter has been reported by Cummins (1921)
2. Specimens could be taken at any time through the summer at the water table; the deepest evidence of burrowing was one crayfish collected at 4 feet under the pond, 1 month after drying

Fall:

1. With refilling of the pond in the fall, a 2-week lag preceded the first appearance of crayfish; if refilling does not occur until winter, crayfish do not emerge
2. In November, 1947, *C. fodiens* were active under 2 inches of ice
3. With heavier ice coverage, all crayfish disappeared from the ponds

GEOGRAPHIC DISTRIBUTION

The range of *C. fodiens* extends from Illinois, Indiana, and Ohio up into Michi-

gan and Ontario (Hobbs, 1948). The usual habitat is that described in the present study, namely, in small mud-bottomed ponds. Cottle (1863) was impressed by the swamp habits and ability to burrow; succeeding workers have corroborated his observations.

The geographic distribution of *O. propinquus* is very similar to that of *C. fodiens*, although it extends farther to the north into Wisconsin and east into New York (Van Deventer, 1937). Over its entire range it is found in rivers, smaller streams, and lakes. Shelford describes it as a "clear-water" form (1913); it is not found in small ponds.

DISCUSSION

Consideration of the ecological relationships between these crayfish and their environments reveals the complexities peculiar to this particular situation and the complexities characteristic of such an ecological relationship generally. Certain questions must be considered: What is the nature of environmental action? Does it act to restrict the resident species, or does it act as a barrier to the foreign species? Is the environmental action constant or seasonal? Is one factor critical, or is the action a result of a complex? A further logical possibility remains: perhaps the environmental action took place historically in the original dispersion of each species to its present range and is at present not important.

The distribution of the stream and pond crayfish is certainly a function of present, as well as past, environmental action. At the point of study, the two habitats are actually connected by a brook for 3 months of the year. In the spring months this brook is populated with young *C. fodiens* down to the confluence with the stream. An occasional specimen was taken from Tinley Creek at this point. No specimens of *O. propinquus* were collected in the brook. With this corridor of exchange open a large part of each year, it is certain that the respective environments are currently acting in some way to prevent dispersal into the reciprocal habitats.

An examination will be made of each environmental factor studied to determine in what ways they may be acting differentially on the two populations.

ACTION OF CURRENT

Water movement is characteristic of the stream, and its absence of the pond. The inference may be made immediately that the difference in crayfish fauna is related to this environmental difference.

This environmental action may be considered as a restriction or barrier; the presence or absence of water movement may be necessary in the life of either crayfish species, or either of these species may be unable to tolerate this aspect of the foreign habitat. It can be readily determined in this instance from the field data. *Orconectes propinquus*, the stream form, has often been taken from lakes, and the results of a natural experiment have been observed by the author in the case of an impounded stream. In twenty-five years the population of *O. propinquus* in this artificial lake appears to be as well established as those in the stream above and below. Flowing water is not necessary in the life of this species, nor is the species intolerant of standing water.

It is equally true, on the basis of field data, that *C. fodiens* is tolerant of slowly flowing water. Records for Dead River (Zion, Ill.) and Tinley Brook support this. However, the former is a partially ponded river, and the latter is connected to ponds.

Two aspects of the problem remain: magnitude of water movement and the

time of breeding in the two crayfish species. A tolerance by *C. fodiens* of slowly moving water is indicated; at its maximum, the rate of current in Tinley Brook did not exceed 30 cm/sec. The time of release to free living of early instars may be an important further consideration. The young of the pond form are released in March, the time of maximum flood in the stream. Those of the stream species are not released until May, after complete subsidence of flood conditions. This time difference in the release of young may be critical.

ACTION OF TEMPERATURE

The water temperatures of Tinley Creek and Tinley Pond I were similar except during early summer; just prior to summer drying, the pond temperatures were higher than those of the stream. If high temperature is acting differentially on the two species, it can be only as a barrier to invasion of the pond at this time by the stream species.

ACTION OF WATER LEVEL

The summer drying of the pond is the most dramatic difference between the two habitats. The burrowing habit of *C. fodiens* undoubtedly enables it to survive; records of burrowing in mud by *O. propinquus* are wanting. *Cambarus fodiens* is not restricted to temporary ponds, but *O. propinquus* is barred from even permanent small ponds. It appears, then, that ability to burrow might be critical for survival in temporary ponds but not in permanent ponds.

Correlative with the burrowing habit must be an ability to tolerate periods of exposure to air. There are indications that *C. fodiens* has this tolerance. Since these crayfish have been seen at the surface of burrows after pond drying, they are then evidencing such a tolerance. This is further documented by a report of overland migrations; high humidity and warm nights were found to be favorable for this terrestrial activity (Cummins, 1921).

ACTION OF THE SUBSTRATUM

A direct relationship exists between mud and rock bottom and the distributions of *C. fodiens* and *O. propinquus*. The former is not found in standing waters with sand, gravel, or rock bottoms but is apparently restricted to a mud substratum. *Orconectes propinquus* is not found in flowing waters if it is a predominantly silted bottom, but appears to be restricted to gravel- and rock-bottomed streams and lakes. It is probable that substratum and species are related causally.

ACTION OF CHEMICAL FACTORS

Dissolved oxygen concentration is generally considered an important ecological factor in aquatic environments. It would appear to be so in the present case. The decrease in oxygen content of the pond is very marked, approaching zero just before drying; stream recordings show continuous saturation. If this factor is acting differentially, it must be as a barrier to the stream form, *O. propinquus*. It may not, however, be assumed that *C. fodiens* can tolerate water approaching zero in oxygen content; at these times burrowing activity is noted. Jewell (1920) reports *O. propinquus* leaving the water and dying on the banks of the Sangamon River at Decatur, Illinois, during a period of low oxygen level.

The calcium carbonate content of the stream was found to be consistently greater than that of the pond. This substance is utilized in the formation of exoskeleton and alimental structures; its lower concentration in ponds could be important as a limiting factor for *O. propinquus*. Since the pond water is not

classified as "very soft," the importance of the hardness factor is questionable.

ACTION OF THE BIOTIC ENVIRONMENT

The included vegetation of the two habitats under consideration differs greatly. The pond is essentially vegetation-filled, while the stream has a sparse bottom flora and marginal emergent vegetation. Each habitat contains a characteristic animal community similar in organization but differing in detail. How may this affect the feeding habits of, and predations on, the two crayfish populations?

Crayfish are omnivorous and scavenging in their feeding behavior. Stomach analyses were made on thirty-six adult crayfish. The contents of *C. fodiens* were comprised largely of plant fragments; a majority contained seeds of the summer grasses. Animal contents were largely insect fragments. In addition, one stomach contained crayfish appendages, one an entire *Asellus sp.*, and one an entire young salamander larva.

Most of the *O. propinquus* stomachs contained filamentous plant fragments which were probably algal; other plant material and seeds were found. Five stomachs contained insect fragments, three contained mayfly naiads, two had stonefly naiads, two had cranefly larvae, one a possible chironomid larva, and one a possible mosquito larva.

These examinations corroborate the evidence of omnivorous feeding habits, the food apparently being dictated by availability. No essential difference between species was noted, and available food appears not to be a limiting factor.

As with available food, the action of predation and parasitism seems unimportant. Lagler (1944) cites specific vertebrate predators on the crayfish of Michigan; birds and fish were found to be prime predators; certain amphibians, turtles, and snakes also eat crayfish. In a review paper on the use of crayfish as food by vertebrate predators, Penn (1950) cites fourteen fish, one salamander, and four turtle species utilizing *O. propinquus* as prey. No data are presented for *C. fodiens*. These predators are common to both habitats, with the exception of fish, restricted to the stream, and salamanders, to the pond. The principal parasite, the Branchiobdellid worm, is ubiquitous on crayfish. However, Goodnight (1940) considers them neither truly parasitic nor host-specific.

SUMMARY OF PART I: FIELD STUDIES OF *C. fodiens* AND *O. propinquus*

1. The usual habitat locality of *C. fodiens* is the small, mud-bottomed, often temporary pond of the Great Lakes region. *Orconectes propinquus* is typically found in rock-bottomed, clear streams and in lakes. There is no habitat overlap.

2. Certain ecological factors of a small stream and an adjacent pond were studied over a period of four breeding seasons. The stream tended toward environmental constancy—in depth, rate of current, temperature, oxygen content, high calcium carbonate content, and absence of stagnation. The pond tended relatively toward the environmental extremes of summer drying, absence of water movement, high summer temperatures, low summer and winter oxygen content, low calcium carbonate concentration, and summer stagnation.

3. The life-histories and geographic distribution of *C. fodiens* and *O. propinquus* were outlined.

4. The possible differential action of the environments on the two crayfish populations was discussed: (*a*) Cited as possible limiting factors to the stream species were the summer drying, decreased oxygen content, muck substra-

tum, high temperature, and low calcium carbonate content of the vernal pond. (*b*) Only the high spring current and possibly the rock substratum of the stream appear probable as barriers to the pond species. The difference in time of hatching may be related to the presence and absence of the spring floods.

II. Comparative Physiology and Behavior

Based on field data, certain inferences were made concerning the environmental action of pond and stream on the contained crayfish populations. The acceptability of these inferences would be greatly enhanced by demonstration of specific physiological reactions. An endeavor was made to examine, test, and evaluate the physiological and behavioral characteristics of the two ecologically divergent crayfish species. A series of experiments was designed to test tolerance to the environmental extremes of the stream and temporary pond. Each of the experiments was constructed to facilitate comparative analysis of reaction to one factor; they were not designed as comprehensive studies of physiological or behavioral processes. Differential mortality or behavior was interpreted as evidence of real difference in reaction to the one factor. The median death of the experimental series from each species was considered the most reliable index; in most instances relatively small numbers were used, and several replicates were therefore necessary. In each experiment the species sets were given identical handling, to justify the comparative interpretation. Death was defined as cessation of all macroscopic activity; this included all gross bodily movement, activity of the legs, abdomen, mouthparts, pleopods, antennae, and anal contractions. With approaching death, movement ceased in that order.

Reaction to Transplantation

Transplantations of the stream species, *O. propinquus*, into the pond under ameliorated conditions in the spring and transplantations of the pond species, *C. fodiens*, into the stream under the optimal conditions of early summer were considered basic to subsequent experiments related to specific environmental factors.

Procedure

1. Two large screened cages were placed in each of the two habitats.

2. Ten crayfish, transplant and control, were placed in each and allowed to remain for 1 month. Natural conditions were simulated, including food sources, except for foraging radius.

3. Each transplantation was duplicated.

Results

Both transplant groups survived as well as the controls, in both sets of experiments. Mortality was very low for transplant and control.

These preliminary experiments were crude in their conception, and the numbers were small; however, survival of transplant under optimal conditions is clearly demonstrated. The necessary next procedure was to examine the reactions to the individual factors differing markedly in the two habitats.

Reaction to Actual and Simulated Pond Drying

Field observations revealed extensive burrowing by the pond crayfish, *C. fodiens;* this reaction had been repeatedly noted by earlier investigators. No instance is known in which the stream crayfish, *O. propinquus*, has left the water. A difference in ability to burrow would be expected, and a set of experiments was designed to test this.

PROCEDURE

Field experiments.—1. At the time of summer drying, two shafts were sunk into the pond bottom. These were of wood, measuring 2 feet deep, and 8 × 8 inches wide; the bottom and top were screened. The shaft was filled with bottom muck.

2. Five pond crayfish were placed in one, and five stream crayfish in the other, and were recovered after 3 days.

3. The experiment was duplicated after the water table had dropped to 12 inches below ground. A total of twenty animals was tested.

Laboratory experiments.—1. Two experimental shafts were constructed of metal ends and glass sides. The dimensions were 16 × 16 × 1½ inches. Activity could be observed through the glass sides.

2. The shaft was partially filled with fine mud and covered over with water; the bottom was open, allowing water to drain out slowly in simulation of natural pond drying.

3. Again the animals were recovered after 3 days. Four replicate sets of six crayfish of each species were tested, a total of forty-eight.

RESULTS

In the field experiments all the *C. fodiens* had burrowed within the shaft to the water level. All were active and in apparent health. None of the *O. propinquus* had burrowed; half were dead on recovery.

Of the eight laboratory experimental shafts, the four containing *C. fodiens* were covered with chimneys. All had burrowed; all were active and in apparent health. The four shafts containing *O. propinquus* had no burrows, all animals remaining on the surface. Of these, almost half were dead after 3 days.

The behavioral difference of the two species under similar conditions is conclusively demonstrated. All the pond crayfish burrowed; none of the stream crayfish burrowed. The ecological significance is equally clear; inability to burrow in mud during summer drying is sufficient cause for the absence of *O. propinquus* in temporary ponds. It does not explain why this stream crayfish is barred from permanent ponds, however.

These experiments do not reveal the nature of the stimulus to burrow. In the experimental instance the actual drop in water level is probably acting. In natural condition, all crayfish are gone days prior to actual drying. The stimulus here would seem to be factors other than actual lowering of water. H. H. Hobbs, Jr., calls my attention to the burrowing of *O. propinquus* beneath the stream stones during the winter months. Therefore, inference from these experimental data must be restricted to the ecological situation of summer drying.

REACTION TO DESICCATION

Field observations of nocturnal terrestrial activity of *C. fodiens* and the record of overland migrations indicate an ability to withstand relatively long periods out of water (Cummins, 1921). A test of this tolerance to drying was made under conditions of constant temperature and relative humidity. The comparative index was longevity in hours.

PROCEDURE

1. Six crayfish, three of each species, were placed in each of a series of dry earthenware crocks.

2. These were kept in a constant-temperature room at 20°.5 and 66 per cent relative humidity.

3. All animals were examined at 2-hour intervals, the dead removed, and elapsed time of survival in hours recorded.

4. Three replicate sets of sixty, sixty-eight, and one hundred animals were tested—a total of two hundred and twenty-eight animals.

RESULTS

A significant difference was recorded in survival time between *C. fodiens* and *O. propinquus*. For the pond species, elapsed time ranged from 7 to 19 hours, with a mean of 12 hours. The range for the stream species was between 1 and 15 hours, with a mean of 6 hours. The survival of *C. fodiens* was double that of *O. propinquus* (see Table 2). The difference between the means is significant in each of the three experiments. Probability that the differences were due to chance was < 0.000 for each experiment. These data corroborate the inference based on field reports and observations that *C. fodiens* is to some extent freed from the aquatic habitat.

TABLE 2

COMPARATIVE TOLERATION TO DESICCATION MEASURED AS MEAN LONGEVITY

Expt.	*O. propinquus*		*C. fodiens*	
	No.	Mean Hours	No.	Mean Hours
I.....	30	6.87±.37	30	11.33±.92
II.....	34	5.21±.17	34	12.35±.42
III.....	50	6.90±.50	50	12.64±.51

REACTION TO CURRENT

The rapid flowing of stream waters and the quiet of pond waters are marked contrasts. To test the possible action on crayfish, the reactions of the two species were studied under several rates of current. A field experiment was devised to determine the downstream distance a crayfish might be swept before maintaining position.

PROCEDURE

1. A chute, 400 × 20 × 20 cm., was placed in a stream along the axis of water flow. The bottom of the chute was covered with gravel and stones from the adjacent creek bed to duplicate natural conditions. The ends were screened.

2. Varying rates of current could be obtained by funneling inflowing water with upstream baffle boards. Rate of flow was determined by timing a weighted cork over the distance.

3. Individuals of alternating species were released in the upstream end of the chute at the water surface and facing the current. Distance to settling was recorded in centimeters.

4. A total of 320 trials was made, at current rates of 25, 33, 40, 50, and 57 cm/sec.

RESULTS

A species difference in ability to settle and maintain position was noted at each of the rates of current; at each rate the mean distance at which *O. propinquus* settled was less than that for *C. fodiens*. The variation in the distance traveled was great, and chance appeared to be a large factor. To facilitate analysis of the data, the median distance was calculated for the entire experimental population and designated the mid-point value. The percentage of each species with greater than this value could then be determined and compared. The percentage difference between the two species was determined for each rate of current and tested for significance (see Table 3).

At each rate of current the percentage difference was significant, with a P value of < 0.000 in each instance. The trials at slower current speeds show less species difference than at the higher speeds. At all speeds the stream species was able to settle within the total 400-cm. distance. At currents of speeds less than 57 cm/sec

the pond species was also able to orient satisfactorily, but at the critical current speed of 57 cm/sec the majority of the pond crayfish tested were unable to settle in the length of the chute.

Since the experimental animals were collected at the time of testing, these results could be wholly or partially affected by differential acclimatization to current. As a control, a sample of each species was kept in laboratory tanks with an absence of current and with otherwise identical conditions for 30 days. Fifty crayfish of each species were then tested in a similar chute at current speeds of 30, 40, and 50 cm/sec (see Table 4).

TABLE 3

COMPARATIVE TOLERATION TO VARIOUS RATES OF CURRENT, DETERMINED BY SPECIES PERCENTAGE CARRIED PAST THE MID-POINT OF THE MEDIAN ANIMAL

Expt.	Current (Cm/Sec)	Mid-point (Cm)	*O. propinquus* Per Cent Over	*C. fodiens* Per Cent Over
I.	25	17	25	75
II.	33	75	20	80
III.	40	100	25	75
IV.	50	240	5	95
V.	50	235	5	95
VI.	50	220	5	95
VII.	57	250	5	95
VIII.	57	260	5	95

This laboratory-acclimatized group tended to react in the same way as those taken from the field. In the slowest current speed there was less difference between species than at the higher speeds. The difference in the case of the 30 cm/sec trials was of questionable significance, having a probability of less than 0.10 but greater than 0.05. The critical speed for *C. fodiens* in these experiments was at 50 cm/sec current, 30 per cent being unable to hold in the entire length of the chute. All *O. propinquus* were able to settle within the chute at all speeds. At the upper current speeds the difference between the species was highly significant.

The data from these two sets of observations justify the conclusion that a real difference exists in the tolerance of the two species to current. This is particularly true of current speeds of 50 cm/sec and above. The pond species, *C. fodiens*, displays a threshold beyond which the crayfish tend to be unable to hold to the substrate. This critical current speed is approximately half as great as those of the spring floods in Tinley Creek; therefore, such a rate of flow would definitely be limiting. The less extreme in water flow would appear not to be a limiting factor for the presence of *C. fodiens* except in so far as they might have competed unsuccessfully with better-adapted forms even at these slower currents.

TABLE 4

COMPARATIVE TOLERATION TO VARIOUS RATES OF CURRENT AFTER 1 MONTH IN QUIET LABORATORY TANKS

Expt.	Current (Cm/Sec)	Mid-point (Cm)	*O. propinquus* Per Cent Over	*C. fodiens* Per Cent Over
I.	30	47	42	58
II.	40	145	6	94
III.	50	267	20	80

REACTION TO INCREASED TEMPERATURES

Differences in early summer temperatures of the stream and of the drying pond were observed. The pond waters became tepidly warm at 30°, while the stream never exceeded 20°. Park (1945), reporting student experiments, conducted in classes in ecology over a period of years, found a high relative heat tolerance by three species of pond crayfish. In the present investigation *O. propinquus* and *C. fodiens* were subjected to above natural temperatures to determine relative tolerance.

PROCEDURE

1. Nine 600-cc. beakers in a constant-temperature bath were used as experimental chambers. These were filled with filtered tap water and maintained at 35°.

2. Experimental animals were taken directly from the field in insulated containers to maintain field temperatures.

3. The crayfish were examined for health and were placed in the beakers by pairs, one of each species. Eighteen animals were tested in each of nine replicates, a total of one hundred and sixty-two animals. Newly molted individuals were excluded.

4. The water was aerated prior to the start of the experiment and at frequent intervals, to insure adequate gaseous exchange.

5. Continuous observation was maintained.

6. At death, each crayfish was removed, its sex was recorded, and its weight and the elapsed time of survival were noted.

7. The median death per species comprised the value for the group.

RESULTS

In each of the experiments the median time of death of *C. fodiens* was higher than the median death of *O. propinquus*. The mean of the medians for the former was four times that of the latter. In the individual experiments there was very little overlap between the death times of the two species. A trend may be noted for the survival time to increase with advance in season and consequent increase in natural temperatures. The significance of the differences between medians was tested and the P value of < 0.000 obtained. While these differences are highly significant, the trend toward greater survival with higher field temperatures indicated some degree of acclimatization to increased temperatures (see Table 5).

To pursue the possibility of temperature acclimatization, identical tests were made on animals kept in warm laboratory tanks at temperatures ranging from 18° to 28° for a period of 5–6 weeks.

The tolerance for both species was many times greater. In each of the four replicate experiments the median survival time for *C. fodiens* was greater than that for *O. propinquus*, but the difference in the medians was reduced from four to one to seven to five. This difference between medians of the acclimatized groups was of questionable significance, P equaling 0.09 (see Table 6).

TABLE 5

COMPARATIVE TOLERATION TO INCREASED TEMPERATURE DETERMINED BY MEDIAN LONGEVITY

Expt.	*O. propinquus* Median Death	*C. fodiens* Median Death
I.....	36	77
II.....	43	145
III.....	45	153
IV.....	24	140
V.....	33	180
VI.....	100	282
VII.....	60	124
VIII.....	25	250
IX.....	31	144

Further investigation was indicated by the degree to which both crayfish species could adapt physiologically to increased temperatures. Tests of a different type were made, in which comparative groups were slowly brought to the lethal temperature of 35°. Thirty crayfish of each species were tested. These had been in laboratory tanks for 8 weeks at temperatures ranging from 18° to 28°. The sixty experimental animals were placed in a large, constantly aerated, constant-temperature tank at 28°. The temperature

was raised slowly through the initial 24 hours to 34° and was then maintained between 34° and 35° for a period of 7 days.

Observations were made over 8 days; at the end of 4 days, ten *O. propinquus* and six *C. fodiens* had died. At 8 days, sixteen of each species remained alive.

A tremendous physiological acclimatization to increased temperature is demonstrated. The comparison of these data with those of crayfish taken from the field is striking, the difference in toleration being of the magnitude of several hundred to one. Individuals of both species were able to tolerate temperatures far above the highest recording in the field. Refined and more complete investigation might reveal a somewhat greater range of heat toleration on the part of the pond crayfish, but the stream form's being able to acclimatize to greater than summer pond heat indicates that the latter is probably not a limiting factor in the distribution of *O. propinquus*.

TABLE 6

COMPARATIVE TOLERATION TO INCREASED TEMPERATURE FOLLOWING MAINTENANCE IN LABORATORY TANKS AT ROOM TEMPERATURES

Expt.	*O. propinquus* Median Death (In Minutes)	*C. fodiens* Median Death (In Minutes)
I.....	288	573
II.....	794	1,030
III.....	380	452
IV.....	575	705

REACTION TO DECREASED OXYGEN

Since the dissolved oxygen content of the stream tended to remain near saturation, while that of the pond underwent marked decreases, a test for toleration to decreased oxygen concentration might reveal the nature of the reaction by the crayfish. Park (1945) reported class studies on several pond and stream crayfish; the pond forms were found to be significantly more tolerant. The present investigation is a study of the two species under the action of very low-oxygen concentrations.

PROCEDURE

1. Wide-mouth, 150-cc. bottles were used as experimental chambers.

2. One crayfish was placed in each; air was displaced by nitrogen, and the bottle filled with low-oxygen water. The bottle was stoppered without entrapped air.

3. Bubbling nitrogen through a 5-liter flask reduced oxygen content to near 0.50 cc/l (less than 10 per cent saturation) in the experimental water. A continuous line extended from a nitrogen cylinder through this water to the experimental bottle. A by-pass line permitted direct gas flow. The experimental bottles were filled under nitrogen gas pressure with low-oxygen water from the flask. The experimental water did not come into contact with air.

4. Four water samples were taken and analyzed for oxygen content by the Winkler method. Temperature remained near 20°.

5. Continuous vigil was maintained until all were dead.

6. Time was recorded as each crayfish was covered with water and at death. Median elapsed time per species was the index of tolerance.

7. Five replicate experiments using eighteen animals were made, a total of ninety.

RESULTS

In each of the experiments *C. fodiens* survived longer than *O. propinquus*, the

difference being approximately four times. The differences of the medians are significant, P being < 0.000 (see Table 7). Here also a possible acclimatization effect could be operating, since the pond crayfish were being taken from waters with less oxygen than the stream.

TABLE 7

COMPARATIVE TOLERATION TO DECREASED OXYGEN TENSION AS DETERMINED BY MEDIAN LONGEVITY

Expt.	*O. propinquus* Median Death (In Minutes)	*C. fodiens* Median Death (In Minutes)
I.....	241	1,328
II.....	167	541
III.....	110	423
IV.....	176	365
V.....	312	634

Crayfish which had been in laboratory tanks for 1 month in water at oxygen saturation were tested in three low-oxygen experiments identical to the previous. The results were similar; again *C. fodiens* survived significantly longer than *O. propinquus* (see Table 8).

Carbon dioxide content was determined in ten water samples taken from bottles at the death of the contained crayfish. In each instance an increase in carbon dioxide of less than 3 mg/liter was found. This increase was probably insufficient to affect mortality, and death was probably due to anoxia.

Considering both sets of experiments, it is seen that a real difference in toleration to lowered dissolved oxygen content exists. It is reasonable to infer that the relative intolerance of *O. propinquus* is related to its absence from ponded waters of periodic low-oxygen concentration.

The entire physiological and perhaps morphological problem of cause in toleration differences would necessarily be a complex one and was not here investigated. Cursory examination of gill volume and preliminary measurements of respiration rate revealed no species differences. A number of basic problems underlie; these would include differences in gross activity as well as metabolic rate and the variance of the latter with temperature, oxygen, and carbon dioxide tensions. The problem of respiration regulation would enter here. Gill-surface permeabilities could be involved and perhaps differences in oxygen utilization efficiencies due to diverse enzyme systems or oxygen affinities to blood pigments.

The oxygen consumption of crustaceans has been investigated by many workers. There seems to be consensus on the dependence of consumption on concentration; some reports indicate a certain regulation of respiration at high but not at lower levels of oxygen (Amberson, Mayerson, and Scott, 1925; Helff, 1928; Heistand, 1931; Maloeuf, 1936). Some comparative studies have been made with reference to habitat. Fox, Simmonds, and Washburn (1935) report on the isopod *Asellus aquaticus* found in both swift and quiet waters; the swift-

TABLE 8

COMPARATIVE TOLERATION TO DECREASED OXYGEN TENSION AFTER MAINTENANCE IN OXYGEN-SATURATED LABORATORY TANKS

Expt.	*O. propinquus* Median Death (In Minutes)	*C. fodiens* Median Death (In Minutes)
I.....	118	284
II.....	280	1,802
III.....	146	1,430

water individuals consumed the larger amount of oxygen. These experiments were conducted without acclimatization. Burbanck, Edwards, and Burbanck (1948) did toleration experiments on the Missouri crayfish *C. rusticus* from streams and *C. setosus* from a cave. The stream form died sooner than the cave species while consuming equal amounts of oxygen; their conclusion of difference in metabolic rate does not seem completely justified. The latter authors also report an incomplete oxygen utilization. Working with *C. bartoni* Maloeuf (1936) found the utilization of the "last trace of analyzable oxygen." Only considerable detailed physiological work with an ecological focus will illuminate the problem of cause in variable tolerances.

REACTION TO SUBSTRATUM

On the basis of field sampling, it was concluded that populations of *O. propinquus* tend to be restricted to rock or gravel substrate, while those of *C. fodiens* are restricted to mud bottoms. A "preference" experiment was designed to test for possible differences in reaction to substratum.

PROCEDURE

1. A concrete tank was divided into two equal parts, each half being 30 inches square. One half was covered uniformly with fine gravel, the other was bisected, corner to corner, to give four, equal-area, triangular sections of alternating mud and washed cinders.

2. Twenty animals of the same species were marked with lacquer and placed in 4 inches of water over the experimental bottom, and twenty of the same species were placed in the control tank of uniform gravel bottom.

3. Light, water temperature, and aeration were uniform. The mud and cinder quadrat was uniformly dark to eliminate color "selection."

4. Observations were made over 3 days, not less than 1 hour apart, and records were kept of the location of each animal.

5. The animals used had been kept for a month or longer in the laboratory on sand substrate.

6. Five sets of *O. propinquus* and five of *C. fodiens* were observed, a total of two hundred animals, plus controls.

The purpose of the experiment was to

TABLE 9

COMPARATIVE ORIENTATION TO ALTERNATE MUD AND CINDER SUBSTRATUM OF EXPERIMENTAL TANKS

O. propinquus (Means per Experiment)					*C. fodiens* (Means per Experiment)				
Expt.	No.	Oriented to		Range	Expt.	No.	Oriented to		Range
		Mud	Cinder				Mud	Cinder	
A	20	5.3	14.7	2.0	B	20	11.0	9.0	3.0
C	20	0.9	19.1	3.0	D	20	12.1	7.9	5.0
E	20	4.4	15.6	5.0	F	20	12.8	7.2	6.0
G	20	0.0	20.0	0.0	H	20	12.6	7.4	8.0
I	20	1.4	18.6	3.0	J	20	10.8	9.2	3.0
Sum		12.0	88.0	13.0	Sum		59.3	40.7	25.0
Means		2.4	17.6	2.6	Means		11.86	8.14	5.0

determine whether the distribution in the control, uniformly graveled bottom, differed from the experimental tank, where a differential orientation to mud or cinder was possible (see Table 9).

RESULTS

Distribution of both species in the control gravel-bottomed tank was random. In the experimental chamber a marked orientation to the cinder was displayed by the *O. propinquus* tested. In one thousand recordings of one hundred *O. propinquus*, 88 per cent oriented toward the the cinder areas. The standard error for the difference between the means being 1.5, the probability that this distribution is chance alone is < 0.000. An equal number of *C. fodiens* was observed; 60 per cent were oriented to the mud areas and 40 per cent to the cinder; this was quite consistent in each of the experiments. The standard error of the difference of the means was 0.58, and the probability that even this relatively slight shift was due to chance alone is < 0.000.

The stream crayfish, *O. propinquus*, under experimental conditions demonstrated a marked orientation toward a cinder-over-mud substrate. Since the color factor was eliminated, the reaction to the environment was probably tactile. In the natural condition this species is restricted to rocky or gravely bottomed streams and lakes. No actual physiological intolerance to mud has been demonstrated, but the behavioral reaction is clear; this may be related to the distribution.

The pond crayfish, *C. fodiens*, demonstrated under these conditions a very slight but consistent orientation toward the mud over the cinder substrate. It seems less likely that this "preference" has barried this species from rocky or gravely bottomed situations.

SUMMARY OF PART II

1. Transplantation experiments to the foreign habitat were conducted. *Cambarus fodiens* placed in moderately flowing water and *O. propinquus* placed in a pond during optimal spring conditions survived in apparent health.

2. Observations were made on behavioral reaction to summer drying of the pond. In field and laboratory experiments, *C. fodiens* burrowed in each instance, while *O. propinquus* burrowed in none.

3. On exposure to drying, survival of *C. fodiens* was found to be twice that of *O. propinquus*.

4. When subjected to various rates of current, *O. propinquus* demonstrated a significantly greater ability to maintain position than did *C. fodiens*. At rates of current less than those of spring flood, *C. fodiens* was unable to hold to the substratum.

5. When subjected to minimum lethal temperatures, *C. fodiens* demonstrated greater tolerance than *O. propinquus*. Such a high degree of acclimatization of both species was found as to make ecological inference dubious.

6. Greater toleration to reduced oxygen was displaced by the pond species than by the stream species.

7. In an experiment designed to test orientation to different substrate textures, *C. fodiens* exhibited a slight "preference" for mud, while *O. propinquus* oriented very strongly toward a cinder bottom.

III. An Ecological Evaluation

The present distribution of *C. fodiens* and *O. propinquus* is necessarily a function of previous as well as current environmental action. The two species are from divergent lineage, with a common glacial and postglacial local history. The

present ecological isolation appears to be a result of a habitat selection by the populations of these species of genera previously well differentiated. Since the present distributions are geographically similar but ecologically different, it was thought that investigation of presently acting environmental conditions and the physiological characteristics related to these conditions would aid in understanding that distribution.

The range of physiological tolerances described has dictated the nature of the environment in which each species may survive. In overland movement the pond species must encounter streams; where these are sluggish and mud-bottomed, survival is possible. In swift streams any *C. fodiens* are eventually eliminated by subtle or direct environmental action. Conversely, in the postglacial movement of *O. propinquus*, invasion of ponds has been possible by direct water routes, but any such invaders would be unable to tolerate one or the complex of pond conditions. The factors which have determined the present distribution are continuing to do so at the periphery of the range and, elsewhere, are acting to maintain that distribution.

In Part I the differences between the adjacent lotic and lentic habitat were described and found to be expressed in terms of substrate, water depth, movement, temperature, and oxygen content. *Cambarus fodiens* was found to occupy the local extreme in standing waters and *O. propinquus* the extreme in running waters. From the comparative physiological studies of Part II, each species was found to be totally adapted physiologically and behaviorally to its characteristic habitat. One exception was noted; physiological reaction to environmental differences in temperature was confused by a wide range of acclimatization of both species. Is each of these factors sufficient to restrict one or the other of the crayfish to its particular habitat? Is any one or all of the factors necessarily acting as a barrier to the alternate habitat?

THE POND SPECIES, *C. fodiens*

For *C. fodiens*, it is clear that the important stream characteristic of water movement acts as a limiting factor. Their presence in slowly moving waters, in addition to the experimental evidence, indicates a certain critical rate of flow as the critical factor of that barrier. For mature crayfish this rate was approximately 50 cm/sec. It is possible that a rate of current less than this may not actually dislodge the animals but may reduce their chance of survival in competition with the other crayfish. In this connection it will be remembered that the brood season of *C. fodiens* is at a time of high stream flood, whereas the season for *O. propinquus* is after the flood period.

Cambarus fodiens displayed a slight but consistent "preference" for a mud substrate. This may reflect subtle behavioral or structural adaptation. In the field they dart rapidly, tail first, into the soft muck of the pond bottom when disturbed. Other stream characteristics appear not to be limiting. Oxygen saturation, constancy of water level, and lower summer temperatures may be discounted as barriers to stream survival.

The specific physiological and behavioral adaptations to the pond habitat seem to be necessary if the past as well as as present needs are considered. All stagnant, mud-bottomed ponds require the fauna to be tolerant of the chemical conditions of stagnancy, particularly to reduction in oxygen content and to the muck substrate. *Cambarus fodiens* was found to have these tolerances. In addition, the elaborate reactions to summer drying are necessary to survival in tem-

porary ponds, though not in those which are water-filled the year round. But these same attributes could be considered essential for successful invasion of large areas by semiterrestrial movement.

THE STREAM SPECIES, *O. propinquus*

A converse situation applies to *O. propinquus;* the barriers to survival in temporary ponds are many. In order of importance are the factors of summer drying, stagnant chemical conditions, principally decreased oxygen, and the mud substrate. Behavioral maladaptation of this species to burrowing in mud has been demonstrated. The stagnant factors are less important in temporary ponds, since they are manifested concomitantly with summer drying and winter freezing. A marked preference for stony over a mud substrate is a real one, though not actually a demonstrated intolerance; in general, the species is not found on mud. The absence of water movements is not a limiting factor.

In ponds with a permanent body of water the inability to burrow is negated as a pertinent intolerance. Here the factors of stagnation effects and substratum are the probable barriers. Again, the avenues of dispersal must be considered; only those ponds which are directly connected to streams could be available to stream population. These are probably few.

The only immediate restriction of *O. propinquus* to the stream is the constant presence of water; they cannot survive in intermittent ponds or streams. The behavioral adaptations to swiftly flowing water are necessary to life in streams but not to maintenance of the population in the lakes where this species is frequently found.

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MODIFICATIONS IN THE DIURNAL PIGMENTARY RHYTHM OF UCA EFFECTED BY CONTINUOUS ILLUMINATION[1]

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THE diurnally rhythmic color change of the fiddler crab, *Uca*, has been described by Abramowitz (1937). The change from the dark coloration characteristic of the day phase of the rhythm to the light coloration characteristic of the night phase continues when the animals are maintained in constant darkness. These color changes are effected by changes in the extent of dispersion of pigments within the chromatophores of the animal. Removal of the eyestalks of *Uca* results in a concentration of black pigment such that the animals normally remain in a lightened condition. Injection of extracts of the sinus gland causes immediate, though temporary, dispersion of the black pigment and, thereby, darkening of the animal (Brown and Scudamore, 1940). It was concluded by Abramowitz (1937) that the day phase of the diurnal rhythm of *Uca* is the result of the presence in the blood of a black-dispersing chromatophorotropin originating in the eyestalks and that the night phase is the result of the absence of this chromatophorotropin from the blood.

Brown (1944) stated that extracts of the central nervous system are effective in dispersing the black pigment of destalked *Uca*. This work was elaborated by Sandeen (1950), who showed that the central nervous system is the source of both a black-dispersing substance and a white-concentrating substance. The black-dispersing substance was shown to be antagonistic to the white-concentrating substance, and it was suggested that the antagonism might be mutual. This is the first described antagonism between two crustacean chromatophorotropins whose primary action is upon different pigments, although there are many instances in which the responses of any one pigment have been shown to be determined by two chromatophorotropic substances, one effecting a concentration and the other a dispersion of the pigment. It was suggested by Brown and Webb (1948) that a concentrating substance might be responsible for the night phase of the rhythm of *Uca*, although the experimental data available at the time could be interpreted in terms of one hor-

[1] This investigation was supported by a research grant from the graduate school of Northwestern University.

mone, namely, the black-dispersing one.

A metabolic basis of the diurnal rhythm of *Uca* was established by Brown and Webb (1948). Further information regarding the characteristics of the control of the diurnal rhythm was obtained from additional studies (Brown and Webb, 1949). During the course of the latter investigation, *Uca* were exposed to constant illumination, and the state of the chromatophores was recorded at four times during each day. The data were interpreted as indicating that there was a gradual reduction in the amplitude of the rhythm, followed by an inhibition of rhythmicity in the chromatophore changes which were observed. It was possible to reverse the daily cycle by illuminating the animals at night and exposing them to darkness by day. Such treatment caused concentration of the black pigment to occur during the day and dispersion to occur at night. This reversed rhythm was found to be persistent when the animals were placed in constant darkness. It was suggested that the time required for reversal of the rhythm was dependent upon the intensity of the illumination, since it was found that reversal appeared to be effected more quickly when higher light intensities were used.

It was shown by Brown and Sandeen (1948) that factors other than diurnal rhythmicity are operative in the normal changes of coloration of *Uca*. An increase in total illumination results in an increased dispersion of both black and white pigments. In addition to this response to total illumination, there is also a response to background, such that the black pigment is more concentrated on a white background than on a black background, other factors equal.

By shielding portions of the legs of eyestalkless *Uca*, Brown, Guyselman, and Sandeen (1949) were able to show that the chromatophores could act as independent effectors, since the shielded chromatophores did not show the dispersion of pigment which occurs when destalked *Uca* are exposed to strong illumination.

The present series of experiments was begun in order to determine the characteristics of the influence of constant illumination upon the diurnal rhythm of *Uca*. Since previous work had suggested that constant illumination for a period of several days inhibited the rhythm, it was of interest to determine whether there was a simple relationship between the light intensity and the time required for inhibition. In addition, the experiments were designed to determine the minimum illumination in which inhibition occurred. During the course of the experiments, however, it was found that complete inhibition did not occur when light intensities up to 250 ft-c. were used. The amplitude of the rhythm in constant illumination was found to be a function of the intensity. It then became of interest to determine the mechanism of this action by light.

MATERIAL AND METHODS

The experiments to be described were conducted at Woods Hole, Massachusetts, during the months of July and August. The experimental animals, *Uca pugnax*, were collected near Woods Hole. The crabs for any given experimental series were collected on the day the experiment began. The crabs were maintained during the experiments in pans containing sea water of a depth of about 1 cm.

The chromatophore activity of these animals under different conditions of illumination was followed by determining the average stage of the pigment in the melanophores in any given experimental group. The Hogben and Slome (1931) scale for describing the degree of disper-

sion of pigment was used. On this scale stage 5 indicates complete dispersion; stage 1 indicates complete concentration, and stages 2, 3, and 4 indicate intermediate degrees of dispersion. In making these determinations the anteroventral surface of the third or fourth walking leg of each animal was observed with the aid of a dissecting microscope, and an estimate was made of the average stage of the pigment in the melanophores. The average stage of the black pigment for all animals in any given group was then calculated.

In studying the influence of constant illumination on the amplitude of the diurnal rhythm, the average chromatophore stages were determined at 1:00, 4:00, 7:00, and 10:00 A.M., and at 1:00, 4:00, 7:00, and 10:00 P.M., for the duration of the experiment.

Groups of twenty animals each were used. Usually, during the course of an experiment some of the animals died. The average number of animals remaining in a group at the end of the several experiments was 17.6.

In a final group of experiments the responses of the melanophores to changes in illumination at several periods in the diurnal cycle were determined. In these experiments the rates and directions of melanophore response to changes from light to darkness and from darkness to light were made at four times during the diurnal cycle. These responses required determination of average chromatophore stages at intervals of 15 minutes.

The light sources utilized in all the experiments were the ordinary fluorescent lamps of the laboratory.

RESULTS

EXPERIMENT I—DIURNAL RHYTHM IN CONSTANT ILLUMINATION

Series I, black background.—Six groups of twenty animals each were maintained in black pans under conditions of constant illumination. Light intensities of 2, 10, 25, 40, 100, and 250 ft-c. were used. The chromatophore activity of each group was followed for 7 days.

Figure 1 represents the data obtained for the group which was maintained at an illumination of 2 ft-c. The average chromatophore stage is plotted on the ordinate. The white and black rectangles on the abscissa and the corresponding ones at the top of the graph represent time. The white rectangles represent the period from 6:00 A.M. to 6:00 P.M., while the black rectangles represent the period from 6:00 P.M. to 6:00 A.M. Examination of the graph shows that a diurnal rhythm persisted for the duration of the experiment. Although the maximum amounts of concentration and dispersion vary throughout the experiment, there is no indication of any trend toward either a decrease or an increase of the amplitude of the rhythmical color change.

The data for the second group, which was maintained at an illumination of 10 ft-c., are presented in Figure 2. It can be seen that a persistent diurnal rhythm was maintained by this group of animals also. Again there does not appear to be any trend toward increase or decrease of the amplitude of the daily cycle. The concentration which occurred during the night phases can be seen to be of a lesser degree than that characteristic of the group maintained at the 2 ft-c. illumination.

Figure 3 presents the data obtained for the third group, which was maintained at an illumination of 25 ft-c. The rhythm of this group would appear to be more variable than those of the previous groups with respect to the maximum amount of concentration which occurred during the several cycles, as well as the time during the night phase at which the maximum concentration was observed.

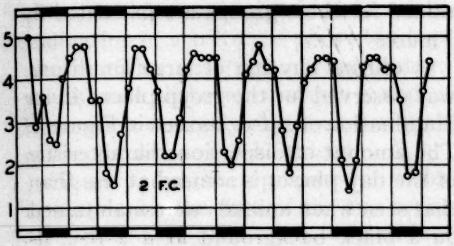

FIG. 1

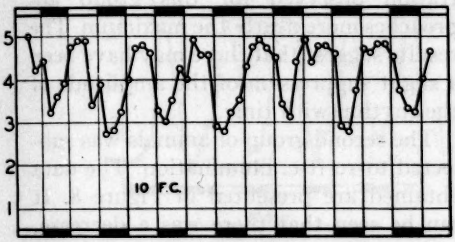

FIG. 2

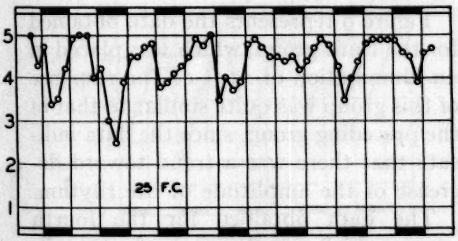

FIG. 3

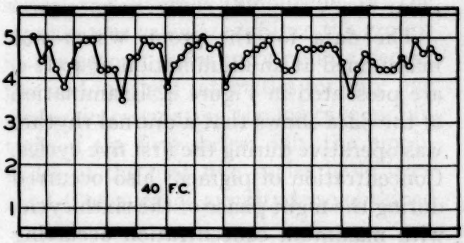

FIG. 4

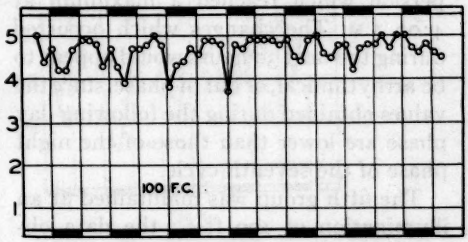

FIG. 5

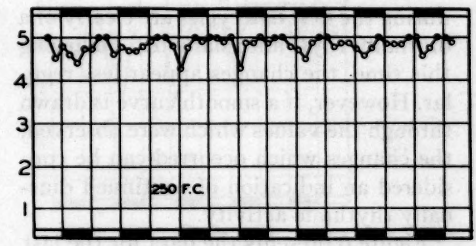

FIG. 6

FIGS. 1–6.—The diurnal rhythms of groups of *Uca* maintained on a black background (Ser. I) in continuous illumination. The light intensities were, respectively, 2, 10, 25, 40, 100, and 250 ft-c. (See text for interpretation of figures.)

However, neither of these variable factors appears to follow a trend in any one direction, and a persistent diurnal rhythm can be followed throughout the experiment.

The data for the group which was maintained at an illumination of 40 ft-c. are presented in Figure 4. Examination of the data shows that a diurnal rhythm was operative during the first five cycles. Concentration of pigment also occurred during the night phase of the sixth cycle, with maximum concentration occurring at 10:00 P.M. This was followed by a dispersion which reached a maximum at 4:00 A.M. The changes which occurred during the next 30 hours would appear to be arrhythmical, or out of phase, since the values obtained during the following day phase are lower than those of the night phase of the seventh cycle.

The fifth group was maintained at an illumination of 100 ft-c.; the data obtained are presented in Figure 5. The chromatophore changes which occurred during the first five cycles are clearly of a diurnally rhythmic character. Following this time, the changes appear less regular. However, if a smooth curve is drawn through the values which were observed, the changes which occurred can be considered an indication of continued diurnally rhythmic activity.

Figure 6 presents the data for the last group of animals in this series. This group was maintained at an intensity of 250 ft-c. The average chromatophore stage observed at all the readings was between 4 and 5. However, careful study indicates that there is a low-amplitude expression of diurnal variation. Although the changes which occurred are small, one or more values obtained during the night phases represent a small degree of concentration when compared with the values observed during the day phases.

Series II, white background.—Groups of twenty animals each were maintained at illuminations of 2, 10, 25, 40, 100, and 250 ft-c. for 5 days. White-enameled pans were used as containers. The data obtained for these groups are presented in Figures 7–12.

A diurnal rhythm of large amplitude was observed for the group placed in an illumination of 2 ft-c., as seen in Figure 7. The amount of dispersion characteristic of the day phases is somewhat less than that seen when animals were maintained on a black background at a 2 ft-c. illumination, while the amount of concentration observed for this group approaches more nearly the maximum. The results suggest that there may have been a slight suppression of the amplitude of the rhythm with time.

The second group of animals was subjected to 10 ft-c. illumination. The data obtained are presented in Figure 8. It can be seen that there was a decrease, with time, of the amplitude of the rhythm.

Figure 9 represents the data obtained for the third group, which was placed in an illumination of 25 ft-c. The response of this group was quite similar to that of the preceding group, since the data indicate that there was a trend toward decrease of the amplitude of the rhythm.

The data obtained for the fourth group, which was maintained at an illumination of 40 ft-c., are presented in Figure 10. The maximum amount of concentration occurred during the second cycle. This was followed by a progressive decrease in the amount of concentration which occurred, resulting again in a suppression of the amplitude of the rhythm.

Figure 11 presents the data for the group which was placed in an illumination of 100 ft-c. The concentration which occurred during the second night phase was considerably greater than that which occurred during the first. During the re-

maining time there was again a progressive decrease in the amount of concentration which occurred.

The chromatophore activity of the last group, which was maintained at an illumination of 250 ft-c., is plotted in Figure 12. The observed chromatophore values were all between stages 4 and 5. However, a diurnally rhythmic variation of low amplitude can be traced.

Figure 13 has been prepared in order to summarize the data of the two series with regard to the relationship of the amplitude of the diurnal rhythm to the light intensity at which the crabs were maintained. For each group in the two series the average amplitude of the rhythm was calculated. Since the groups in the second series were maintained at the various light intensities for only five cycles, in calculating the average amplitude of the first series, only the amplitudes of the first five cycles have been used. The average amplitude so obtained

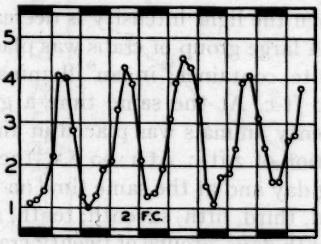

FIG. 7

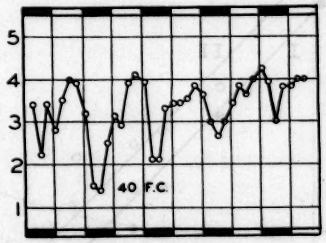

FIG. 10

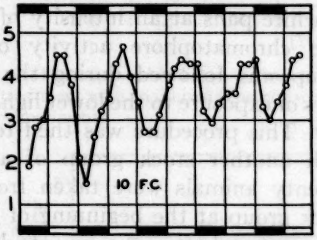

FIG. 8

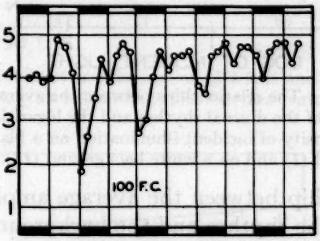

FIG. 11

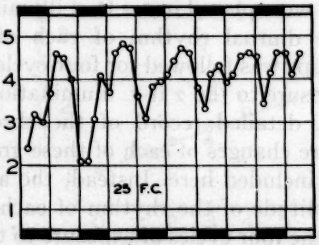

FIG. 9

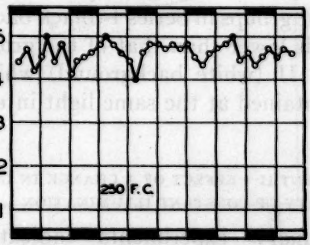

FIG. 12

FIGS. 7–12.—The diurnal rhythms of groups of *Uca* maintained on a white background (Ser. II) in continuous illumination. The light intensities were, respectively, 2, 10, 25, 40, 100, and 250 ft-c.

is plotted against the logarithm of the light intensity to which the group was exposed. Curve *I* represents the values obtained for Series I, while Curve *II* represents the values obtained for Series II. It can be seen that there is an inverse relationship between the average amplitude of the rhythm and the light intensity to which the crabs are exposed. This graph also shows that there is a definite

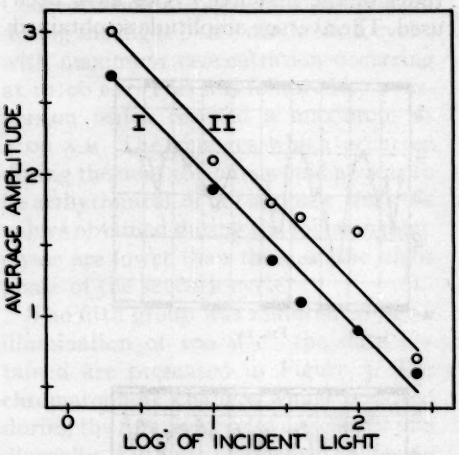

FIG. 13.—The relationships between the average amplitude of the diurnal rhythm and the logarithm of the intensity of incident illumination on a black background (*I*) and on a white background (*II*).

relationship between the average amplitude of the rhythm and the background on which the animals are maintained, since each of the average-amplitude values for the groups in Series I (black background) is lower than that of the group in Series II (white background) which was maintained at the same light intensity.

EXPERIMENT II—EFFECT OF A CHANGE IN INTENSITY OF CONSTANT ILLUMINATION

Preliminary experiments indicated that a marked increase in the amplitude of the rhythm occurred when animals which had been maintained for some time at an illumination of 250 ft-c. were placed in a 2 ft-c. illumination. However, the rhythm of such animals was less, at the lower light intensity, than that of animals maintained in a 2 ft-c. illumination without previous treatment with constant illumination of 250 ft-c. intensity. It was believed that further information regarding the increase in amplitude following a lowering of light intensity would be of value. An experiment was designed to determine whether the length of exposure to a higher light intensity affects the amplitude of the rhythm when the light intensity is decreased.

A large group of crabs was placed in a white container, in an illumination of 250 ft-c. At the same time a group of twenty animals was placed in an illumination of 2 ft-c. At 1:00 P.M. the following day and at the same time on the second, third, fifth, seventh, tenth, and sixteenth days, groups of twenty crabs were removed from the stock group being maintained at 250 ft-c. and were placed in white pans at an intensity of 2 ft-c. The chromatophore activity of each group was followed during the first 4 days of exposure to the lower light intensity. This procedure was then repeated with another stock group of animals. Twenty animals were taken from this stock group at the beginning of the experiment and after 1, 2, 3, 4, and 5 days of exposure to the 250 ft-c. illumination and were placed in a 2 ft-c. illumination. The diurnal rhythm of each of these groups was followed for four cycles after exposure to the 2 ft-c. illumination.

A detailed record of the chromatophore changes of each of these groups is not included here. Instead, the average amplitude of the rhythm of each group for the four cycles of exposure to the low light intensity was calculated. Figure 14 gives these values, plotted against the number of days of previous treatment with 250 ft-c. illumination. It can be seen

that there is an inverse relationship between the average amplitude of the rhythm at the low light intensity and the length of previous treatment with constant illumination of high intensity.

Further analysis of the data indicated clearly that the gradual reduction in the amplitude of the rhythm was effected by a gradual decrease in the amount of concentration of pigment which occurred during the night phases. This can be seen in Figure 15, in which is plotted the average maximum dispersion by day and concentration by night of the groups exposed to various periods of high constant illumination.

EXPERIMENT III—IMMEDIATE EFFECT OF CHANGE OF ILLUMINATION

Another type of experiment was designed in order to determine the immedi-

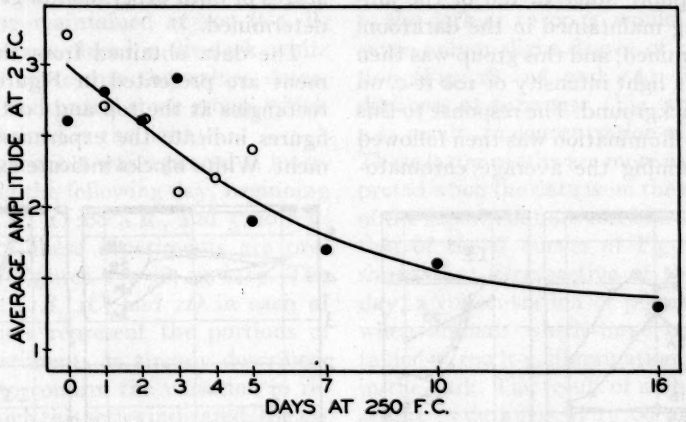

FIG. 14.—The relationship between the average amplitude at an illumination of 2 ft-c. and the days of previous treatment with an illumination of 250 ft-c. Black circles and white circles represent values obtained from two experiments.

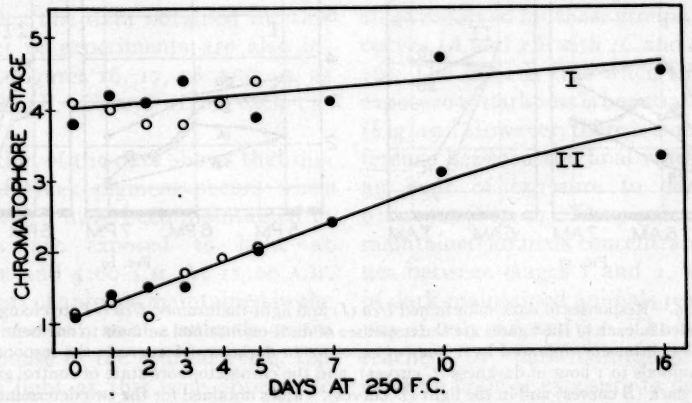

FIG. 15.—The relationships between the days of previous treatment with an illumination of 250 ft-c. and the average maximum dispersion during day phases (*I*) and concentration during night phases (*II*) at an illumination of 2 ft-c. Black circles represent values obtained in one experiment, and white circles those in a second.

ate effect of changes in illumination upon the chromatophores at various periods during the diurnal cycle. The crabs were collected during the morning and were divided into two large groups. One group was placed in a darkroom at 1:00 P.M., while the other was placed at the same time in an illumination of 100 ft-c. The latter group was maintained on a white background. At 11:00 P.M. the average chromatophore stage of ten of the animals being maintained in the darkroom was determined, and this group was then placed in a light intensity of 100 ft-c. on a white background. The response to this change in illumination was then followed by determining the average chromatophore stage of the group at intervals of 15 minutes. At 12:00 P.M. the group was returned to the darkroom. Also at 12:00 P.M. the average chromatophore stage of ten of the animals from the stock group being maintained at 100 ft-c. illumination was determined, and this group was also placed in the darkroom. During the next hour, at intervals of 15 minutes, the average chromatophore stages of both experimental groups were determined.

The data obtained from this experiment are presented in Figure 16. The rectangles at the top and bottom of the figures indicate the experimental treatment. White blocks indicate exposure to

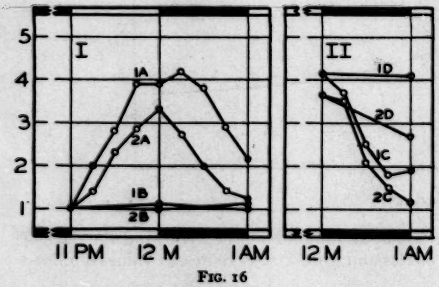

FIG. 16

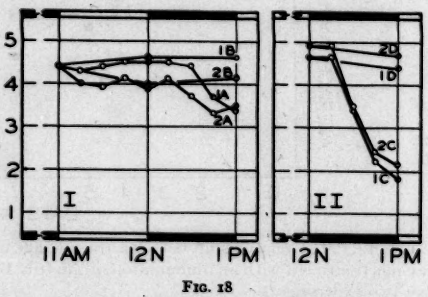

FIG. 18

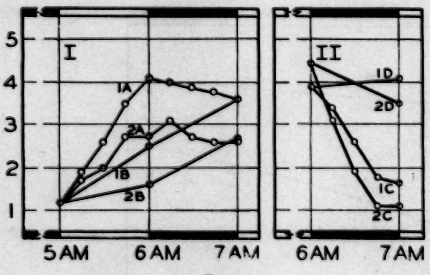

FIG. 17

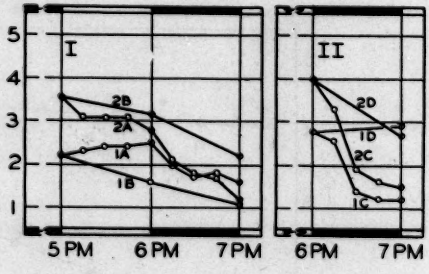

FIG. 19

FIGS. 16–19.—Responses of dark-maintained *Uca* (*I*) and light-maintained *Uca* (*II*) to changes in illumination. Included in each of the figures are the responses of dark-maintained animals to one hour of illumination of 100 ft-c. intensity, followed by 1 hour of exposure to darkness (*A* curves); the responses of light-maintained animals to 1 hour of darkness (*C* curves); and the chromatophore state of control groups maintained in the dark (*B* curves) and in the light (*D* curves). Values obtained for the two determinations made at each of the periods in the diurnal cycle are distinguished as *1* and *2* curves. Determinations were made at 11:00 P.M. and 12:00 M. (Fig. 16), 5:00 A.M. and 6:00 A.M. (Fig. 17); 11:00 A.M. and 12:00 N. (Fig. 18), and 5:00 P.M. and 6:00 P.M. (Fig. 19). The black rectangles on the abscissae and at the top of the graphs indicate exposure of the experimental groups to dark, while white rectangles indicate exposure to an illumination of 100 ft-c. intensity.

100 ft-c. illumination, while the black blocks indicate exposure to darkness. Curve *1A* represents the average chromatophore response observed for the group which was removed from the darkroom, exposed to light for 1 hour, and returned to the darkroom. Curve *1B* represents the values determined for the control stock group which was maintained in the dark. Curve *1C* represents the values obtained when a group from the stock being maintained at 100 ft-c. illumination was placed in the dark, while Curve *1D* represents the values determined for the control stock group, which was maintained in the light.

The experiment was repeated at intervals during the following day, beginning at 5:00 A.M., 11:00 A.M., and 5:00 P.M. Data from these experiments are presented in Figures 17, 18, and 19. The curves *1A*, *1B*, *1C*, and *1D* in each of these figures represent the portions of these experiments as already described. In order to confirm the variation in response which this series indicated, the experiments were continued for an additional day, so that two experiments were performed at each of four times during a daily cycle. The data obtained for this second set of experiments are also included in Figures 16, 17, 18, and 19, as curves *2A*, *2B*, *2C*, and *2D* in each figure.

Inspection of the data shows that dispersion of black pigment occurs when animals which have been maintained in the dark are exposed to light at 11:00 P.M. and 5:00 A.M. At 11:00 A.M. the pigment of animals maintained in the dark is dispersed, as the animals are in the day phase of the diurnal cycle. Exposure to light at this time appears to have little or no effect on the chromatophores. Exposure to light at 5:00 P.M., when the pigment is in an intermediate stage between dispersion and concentration, also does not appear to have any significant effect upon the chromatophores.

It can also be seen that when crabs which have been exposed to 1 hour of light are returned to the dark at 12:00 P.M. (Fig. 16, *1A* and *2A*) concentration of the black pigment occurs. The same treatment of another group at 6:00 A.M. results in relatively little concentration (Fig. 17, *1A* and *2A*). Return to the dark at 12:00 M. would appear to cause only a slight degree of concentration (Fig. 18, *1A* and *2A*). Return to darkness at 6:00 P.M. (Fig. 19, *1A* and *2A*) results in concentration of pigment. These latter results are more easily interpreted when the data from the remainder of the experiment are considered. Inspection of the *C* curves of Figures 16–19 shows that, irrespective of the time of day, a concentration of pigment occurs when animals which have been maintained in 100 ft-c. illumination are placed in the dark. The result of an hour of exposure to darkness at 12:00 P.M. is similar for both light-maintained and dark-maintained animals, as can be seen from inspection of the final chromatophore stage observed for these groups (compare curves *1A* and *2A* with *1C* and *2C* in Fig. 16). The same is true when the hour of exposure to darkness is begun at 6:00 P.M. (Fig. 19). However, there is a marked difference between the final readings after an hour of exposure to darkness at 6:00 A.M. (Fig. 17). The pigment of light-maintained animals concentrated to values between stages 1 and 2, while that of dark-maintained animals reached values of only 2.6 and 3.6. The final value reached by dark-maintained animals after an hour of exposure to darkness at 12:00 M. was about 3.5, while that of light-maintained animals was about stage 2 (Fig. 18). When these comparisons are made, it can be seen that the response to

change from light to darkness depends not only upon the time in the diurnal cycle of the exposure to darkness but also upon the previous history of the animals.

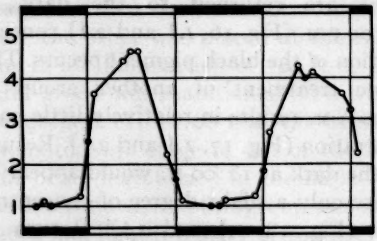

FIG. 20.—Diurnal rhythm of the dark-maintained *Uca* used in Experiment III.

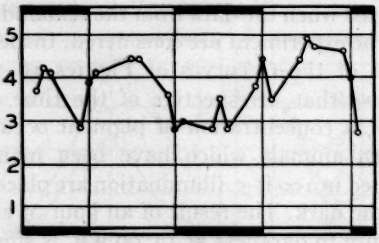

FIG. 21.—Diurnal rhythm of the light-maintained (100 ft-c.) *Uca* used in Experiment III.

The data obtained for the stock groups, which were maintained in constant darkness and constant illumination, are presented in Figures 20 and 21, respectively.

DISCUSSION

Brown and Sandeen (1948) have shown that the response of *Uca* to total illumination is such that, when other factors are equal, there is increased dispersion of the black pigment as the intensity of illumination increases. The data obtained from both illumination series of Experiment I have shown this to be a significant factor in the determination of characteristics of the diurnal rhythm under conditions of constant illumination. Both the maximal degree of dispersion and the maximal degree of concentration are affected. However, the differences in the amplitudes of the rhythms of the groups maintained at the various illuminations on a black background are chiefly the result of the differences in the amount of concentration which occurred during night phases (see Table 1). The average maximum dispersion values for the several groups show that an increase in illumination does result in increased dispersion. The differences are small, however, and relatively unimportant in de-

TABLE 1

MAXIMUM DISPERSIONS AND CONCENTRATIONS, AND RHYTHM AMPLITUDES OF *Uca* BLACK PIGMENT DURING THE DIURNAL RHYTHM IN CONSTANT ILLUMINATIONS OF DIFFERENT INTENSITIES

	Illumination (Foot-Candles)					
	2	10	25	40	100	250
	Series I—Average Values for Five Cycles					
Maximum dispersion	4.7	4.9	4.9	4.9	4.9	5.0
Maximum concentration	2.0	3.0	3.5	3.8	4.0	4.4
Amplitude	2.7	1.9	1.4	1.1	0.9	0.6
	Series II—Average Values for Five Cycles					
Maximum dispersion	4.2	4.5	4.7	4.0	4.8	4.9
Maximum concentration	1.2	2.4	2.9	2.3	3.2	4.2
Amplitude	3.0	2.1	1.8	1.7	1.6	0.7
	Series II—Values for Fifth Cycle Only					
Maximum dispersion	3.8	4.5	4.6	4.1	4.8	4.6
Maximum concentration	1.6	3.0	3.4	3.0	4.0	4.3
Amplitude	2.2	1.5	1.2	1.1	0.8	0.3

termining the amplitude of the rhythm. On a white background these differences are greater. Here the amount of illumination determines the amplitude of the rhythm chiefly by modifying the maximum amount of concentration during the night phases and to a lesser extent the amount of dispersion during the day phases.

It was also shown by Brown and Sandeen (1948) that *Uca* responds to background. This response is such that the black pigment is more concentrated on a white background than on a black background, when other factors are equal. An influence of this factor can be seen in a comparison of the two series (see Figs. 1–12). The most striking difference between these two series is seen in the relationship between amplitude of rhythm and length of time the animals are maintained under constant illumination. In those animals maintained upon a black background there is apparently no significant alteration in rhythm amplitude with time, whereas in the animals maintained upon a white background, the amplitude is typically great at first, becoming gradually reduced over a 3- to 5-day period of continuous illumination. This difference is evident at 2 ft-c., very conspicuous from 10 ft-c. through 100 ft-c., but absent at 250 ft-c.

Concentration during the night phase is augmented by the albedo response to a white background, and this is in all probability the factor responsible for the initially greater amplitude upon a white background. The decrease with time in the amount of concentration which occurs on a white background indicates that there occurs within the organism a gradual reduction in ability to respond to a white background during the night phase. The absence of this gradual alteration which was observed at the illumination of 250 ft-c. may be interpreted as being the result of direct suppression of concentration of the black pigment by the strong illumination, which is antagonistic to the albedo response. Although the initial amplitude difference between groups in the two series tends to disappear, the amplitudes for animals on white background becoming equivalent to those of groups on black (compare data for fifth cycle of Ser. II with average values for Ser. I, in Table 1), an albedo influence still persists. This influence continues as one affecting the magnitude of the maximal dispersion during the day and night phases. Those animals maintained upon black backgrounds continue to have these values consistently equal to, or higher than, those upon white backgrounds.

On a black background, on the other hand, the albedo response is antagonistic to the concentration of black pigment during the night phase of the rhythm. There appears to be no corresponding alteration with time in the ability of the organism to respond to background under these circumstances.

The data obtained from Experiment II suggest an additional effect of continuous illumination upon the color-change system. Animals subjected to higher illumination for a period of time experience an abrupt increase in the amplitude of the rhythm when placed in a continuous illumination of lower intensity. The increase in amplitude which occurs under these circumstances has been shown to be reduced as the animals are maintained for longer times at the higher illumination. The effect of constant illumination must include, therefore, not only a direct and quickly reversible response but also a more gradual and nonreversible one. This gradual response of the color-change system is expressed as a decrease in the ability of the organism to effect concentration and becomes evident

when the direct or immediate suppression of the amplitude of the rhythm by high light intensity is removed. The mechanism which is responsible for this gradual response is possibly the same as that responsible for the gradual reduction of the amplitude of the rhythm of animals maintained on a white background in constant illumination of intensities up to 100 ft-c., as described earlier.

It will be recalled that in Experiment III a concentration of pigment always occurred when animals which were maintained at an illumination of 100 ft-c. were placed in the dark and that concentration did not always occur when animals which had been maintained in the dark, previous to an hour of illumination, were returned to the dark. These experiments clearly show that the response to a change in illumination cannot be explained simply as a primary effect of light, for the response is modified by both the previous treatment of the animals and the phase of the diurnal cycle. It seems most probable that the differences of response are the result of variations of chromatophorotropically active substances, either in the amount stored within glands or in the amount circulating in the body fluids. The only clearly demonstrated black-influencing chromatophorotropin of *Uca* is a black-dispersing one. From the observations on the response of light-maintained animals to a light-to-dark change, it appears reasonable to assume that this stimulus results in a rapid disappearance of the black-dispersing substance from the blood. If this were the only factor involved, there is no evident reason why crabs exposed to 1 hour of an illumination of 100 ft-c. should not respond similarly to a light-to-dark change, since 1 hour should be adequate for photic adaptation of the receptive mechanism of the animals. These animals respond, however, quite differently. The diurnal rhythm of the control animals (see Figs. 20 and 21) would indicate that the blood titer of a dispersing principle was about equally high in the two groups during the peak of the day phases. Despite this apparent similarity, the light-to-dark change at 12:00 M. resulted in almost complete concentration of the black pigment of the light-maintained animals, but very little concentration of the pigment of dark-maintained animals.

Furthermore, if the response to a light-to-dark change involves only the disappearance of the black-dispersing substance, it would appear logical to assume that the rate of concentration would be dependent upon the amount of dispersing substance present in the blood. At 6:00 P.M. (see Fig. 19) the state of dispersion in the dark-maintained animals was less than that of light-maintained animals, suggesting that a lower concentration of dispersing substance was present in the dark-maintained animals at this time. However, the rate of concentration of the dark-maintained animals was less than that of the light-maintained animals.

In view of such difficulties in explaining the response to a light-to-dark change simply as the disappearance of the black-dispersing hormone, it seems reasonable to postulate the existence of a concentrating factor. Such a postulate is not unreasonable in view of the fact that a dual control, including both dispersing and concentrating agents, has been demonstrated for most chromatophores which have been critically investigated. With such a postulate, it immediately becomes simpler to interpret the observed results. Under these circumstances, those animals maintained in continuous illumination could be considered to have throughout the 24-hour cycle a relatively high blood titer of a concentrating factor

which is more or less masked by the dispersing factor. Another possibility is that there could be a low blood titer but considerable storage of the concentrating substance in glands. In either case the concentrating substance would be available for immediate and rapid pigment concentration following cessation of activity of the black-dispersing agent in response to the illumination change.

The animals maintained in constant darkness would appear to possess their highest blood titer, or glandular stores, of the concentrating factor at 6:00 P.M. and 12:00 P.M. (see Figs. 19 and 16) and their lowest titers or stores at 6:00 A.M. and 12:00 M. (see Figs. 17 and 18). This is as one would reasonably expect, judging from the observed chromatophore states at the four periods in the diurnal cycle, were a concentrating factor normally operative in the night phase.

Such a hypothesis of the role of a black-concentrating factor is given further support by the observation that the qualitative response of all the groups in Experiment III to a light-to-dark change was one of concentration of black pigment; only rate and degree of concentration differed among the animals. If it is assumed that animals under all these circumstances are capable of reducing at equivalent rates the blood titer of the black-dispersing principle when subjected to similar stimuli, the most logical explanation of differences in rates of concentration is the presence of different concentrations of a black-concentrating factor, as determined by long-term previous stimulation.

SUMMARY

1. The amplitude of the diurnal rhythm of melanophores of *Uca* exposed to constant illumination varies inversely with the intensity of the illumination.

2. The reduction of the amplitude of the rhythm of *Uca* maintained in continuous illumination of 10–250 ft-c. intensity is primarily the result of a decrease in the amount of concentration which occurs during the night phase.

3. The albedo response is reflected both in the concentration during the night phase, which is greater on a white background than on a black background, and in the dispersion during the day phase, which is greater on a black background than on a white background.

4. The rhythm of *Uca* maintained on a white background in illuminations of 2 to 100 ft-c. intensity is characterized by a decrease, with time, of the amplitude of the diurnal cycle.

5. *Uca* maintained for some time in constant illumination show, upon change to constant illumination of lower intensity, an immediate increase in rhythm amplitude. The amplitude of the rhythm at the lower intensity is a function of the length of the period of exposure to the higher light intensity.

6. A rapid concentration of black pigment always occurs when *Uca* which have been exposed to 18–66 hours of continuous illumination (100 ft-c.) are placed in darkness.

7. The response to a change from light to darkness of *Uca* which have been maintained for 10–58 hours in darkness and then treated with 1 hour of illumination varies with the phase in the diurnal cycle.

8. These responses of *Uca* to light-to-dark changes appear to require for their interpretation the operation of a black-concentrating factor, in addition to the now well-known dispersing one.

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MECHANISMS REGULATING THE REPRODUCTIVE CYCLE IN THE CRAYFISH, CAMBARUS. I. THE FEMALE CYCLE[1]

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I. INTRODUCTION

A. REVIEW OF THE LITERATURE

MUCH research has been carried out on the environmental regulation of reproductive cycles in almost all classes of vertebrates, and in many cases the physiological mechanisms are rather well known whereby breeding cycles are correlated with seasonal changes in such a way that the offspring are produced at a time most favorable for their survival. Many excellent reviews on various aspects of this subject (Rowan, 1938; Brooks, 1939; Wolfson, 1941; Marshall, 1942; Turner, 1948) make further consideration of the problem unnecessary here.

Concerning the same problem among the invertebrate phyla, however, relatively little is known, either as to specific environmental influences responsible for correlation of reproductive activity, or as to hormonal or other mechanisms by means of which these responses are mediated within the organism itself. Rowan, in his above-mentioned review, states that it has been shown for the strawberry-root louse, *Aphis forbesi*, that, under short day-lengths, oviparous females appeared in May instead of the usual time, November. However, since this animal is a root parasite, it appears probable that the effect is a secondary one, with the animals responding to the altered physiology of the plant induced by decreased day-length. Ballard (1942) found in the hydroid *Hydractinia* that both males and females discharge their gametes only when exposure to light of some critical duration follows a previous induction period in darkness.

Among the Arthropoda, several relationships between the gonads and tissues

[1] This investigation is a portion of a thesis submitted in partial fulfilment of the requirements for the Ph.D. degree in biology at Northwestern University, in June, 1951. The author wishes to express her sincere appreciation to Dr. Frank A. Brown, Jr., for his inspiration and helpful criticisms during the course of this investigation.

possessing endocrine functions have been elucidated. Of the insects, for example, it has been shown for the orthopteran *Melanoplus differentialis* that complete removal of the corpora allata from adult females prevents the production of ripe eggs in the ovaries; the eggs appear to be independent of the corpora allata until they attain the stage at which yolk deposition would begin, but absence of the organs after that period causes the eggs to degenerate and become resorbed (Pfeiffer, 1939). A similar situation obtains in several other insect orders. Thomsen (1948), working with the dipteran *Calliphora*, has presented evidence that certain neurosecretory cells in the brain of this animal are responsible for control of the activity of the corpus allatum, at least as concerns its reproductive functions.

An important reproductive function of the crustacean sinus gland was first demonstrated by Panouse (1943, 1944, 1946) in experiments on the shrimp, *Leander serratus*. Removal of the eyestalks or bilateral ablation of the sinus glands alone, during a time of reproductive quiescence in these animals, initiates a period of rapid oöcyte maturation, which may culminate in deposition of apparently normal eggs. Sinus glands implanted in the abdomens of eyestalkless animals prevent such a response. A similar hormonal relationship between the sinus gland and the ovary, with the former acting as inhibitor of yolk deposition in the oöcytes, has been demonstrated for the fiddler crab, *Uca* (Brown and Jones, 1948), and the crayfish, *Cambarus* (Brown and Jones, 1947). In the latter case, eyestalk removal was performed in late June, which is the approximate period during which ovarian maturation is initiated in this form. (For the purposes of this work, ovarian maturation is considered to be yolk deposition and other changes in the oöcytes accompanying this process.) Over the 53-day period covered by the experiment the average dry weights of the ovaries of the eyestalkless crayfish increased linearly with time from approximately 15 to 180 mg., more than a twelvefold increase. During the same period the ovaries of untreated controls increased from 15 to approximately 51 mg. In neither case did egg-laying occur.

Another action of the sinus gland related to reproduction in the crayfish was described by Scudamore (1948), who found that the phenomenon of the delay in spring molt of egg-bearing females is regulated by the action of the molt-inhibiting hormone of the sinus gland.

McVay (1942), in making a study of the chromatophorotropic activities of central nervous tissues in *Cambarus*, encountered an apparent relationship between these tissues and the reproductive cycle of the female, as follows: (*a*) extracts of "brains" of females containing viable eggs have little or no effect on the white chromatophores; (*b*) "brains" of females which have laid eggs or in which the oöcytes are being resorbed produce concentration of the white pigments; (*c*) boiling of the "brain" extract in the inactive condition converts it to the active state; (*d*) "brains" of males always have this factor in the active form.

For reviews of other important functions of the sinus gland and the central nervous system in the physiological economy of the crustaceans see Brown (1944, 1948, 1950).

B. STATEMENT OF THE PROBLEM

The series of experiments presented here represents an attempt to obtain information concerning environmental factors (specifically, light) which may have a regulatory effect on the reproductive cycle of the crayfish and on certain tis-

sues or organs of these animals (specifically, the sinus gland and the central nervous system) through which such a response might be mediated.

The animals used were females of *C. virilis*, ranging in carapace length from 35 to 45 mm., obtained from a biological supply house in Wisconsin. According to information obtained from individuals responsible for supplying these animals, the groups for each experiment were usually shipped immediately after being collected and were thus not subjected to abnormal conditions of light or temperature before the experiments were begun. The animals of this region hibernate during the winter months by burrowing into the banks of streams or the mud of river or lake bottoms and thus pass these months in complete, or nearly complete, darkness. Females lay their eggs sometime in May, depending upon the weather, and the young hatch in June.

II. Light Experiments

A. Materials and Methods

The animals used in these experiments were kept in large tanks supplied with a constantly running stream of tap water. Lighting was achieved by 25-watt, unfrosted, incandescent bulbs mounted on a frame suspended 1 foot above water level; the light intensity at the surface of the water varied in different portions of the tank from 5 to 15 foot-candles. The tanks were covered with black cloth to prevent extraneous light from entering (in the case of the 24-hour and normal-day-length tanks) and to maintain total darkness in the third. Light duration was controlled by automatic time clocks, which, in the one tank, maintained 20 hours of light per day and, in the other, kept a daily photoperiod which corresponded exactly to that of normal day-length. The temperatures of the water in the three tanks were kept similar by regulating the rate of water flow. The animals were not fed throughout the course of the experiment.

In early September, eighty females were placed in each of the three above-described tanks. The condition of the ovaries at this time was ascertained by autopsy of five animals and was found to be that expected for this time of year, that is, the oöcytes measured about 0.6 mm. in diameter and were gray- or brown-green. Specimens were examined histologically; Bouin's fluid was used as fixative, the ovaries were imbedded in paraffin and sectioned at 12 μ, and the sections were stained with Delafield's haematoxylin and acid fuchsin. Yolk granules were abundant in the cytoplasm of the oöcytes, the epithelium was low cuboidal or squamous, and there was very little proliferation of interstitial material (Fig. 1).

The progress of these three groups of animals throughout the course of the experiment, which lasted through June 3, was followed in three ways: observations of blood coloration, gross examination of ovaries at autopsy, and histological examination of ovarian tissue as described above.

B. Results

1. Blood Coloration

At intervals each of the animals in the three tanks was examined, and it was seen that varying numbers of them were showing an orange or red blood coloration, readily observable through the transparent chitin of the ventral surface of the abdomen. Results of these examinations for 20-hour and normal-day-length animals are shown in Figure 14, where percentages of animals demonstrating such coloration are plotted against months. Only sporadic cases of

red blood color were seen among animals in the dark tank.

2. GROSS EXAMINATION OF OVARIES

Specimens of animals with red blood color were sacrificed, and the ovaries were seen to be filled with oöcytes of various sizes and colors, apparently representing different stages of resorption of yolk substances. Larger oöcytes were dark gray or black (an index of beginning degeneration), while the smaller were orange or yellow. In cases where the majority of oöcytes were being actively resorbed, a reddish fluid was seen to lie between them and the membrane of the ovary itself. The intensity of blood coloration is correlated directly with numbers of oöcytes affected; the blood is not colored in specimens in which the majority of oöcytes have reached a certain minimal size or when resorption is just getting under way.

Since the blood color is such an excellent index of the condition of the ovaries of that portion of the crayfish population undergoing extensive yolk resorption and since the animals undergoing this resorption to a degree great enough to cause an obvious shift in blood color were usually in the minority, animals with the normal clear blood were sacrificed at intervals to furnish samples indicative of the state of the ovary in the majority of the group. Figure 13 is a photograph of a series of typical ovaries from the three groups during the course of the experiment. No ovaries are shown for the animals in total darkness beyond February, since there appeared to be no further change in this group. For the sake of comparison with the blood-color graph, this information concerning ovarian size for 20-hour and normal-day-length animals is shown graphically in Figure 15, where length of the ovary in millimeters is plotted against months.

FIG. 13.—Three series of ovaries removed, on the dates indicated, from animals which had been exposed since September 2 to the following experimental conditions: *A*, daily 20-hour photoperiods; *B*, normal day-length; and *C*, continuous darkness.

3. HISTOLOGICAL EXAMINATION OF OVARIAN TISSUE

As the cyclical changes depicted in Figures 13, 14, and 15 proceed, the fol-

lowing sequence of events is seen to occur histologically:

a) ***Resorption of yolk from the oöcyte.***—Large vacuoles appear in the yolk, and the oöcyte epithelium becomes high cuboidal instead of the squamous type usually seen in the late stages of maturation; yolk substances may sometimes be seen

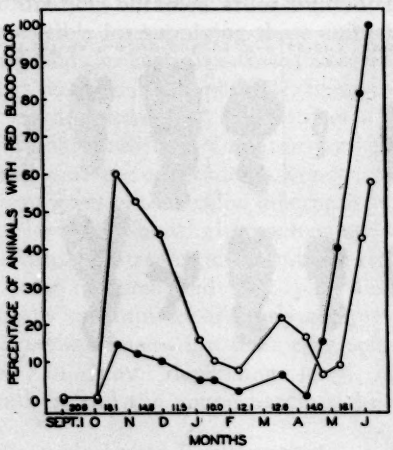

FIG. 14.—Relationship between percentage of 20-hour and normal-day-length animals with red blood color, and time in months. Average water temperatures for each month are indicated. 20-hour: ○; normal-day-length: ●.

in vacuoles within the epithelial cells of the oöcytes.

b) ***Breakdown and removal of the oöcyte cytoplasm itself.***—This process occurs after the yolk has passed from the cytoplasm. Vacuoles appear in the cytoplasm, and the oöcyte begins to collapse. The epithelial cells become high columnar, and their vacuoles are left empty when yolk substances are passed into the blood.

c) ***Further changes.***—As collapse and destruction of the old oöcytes proceed, there is (1) great proliferation of interstitial material and (2) formation of large numbers of new, healthy-looking oöcytes, which, after the attainment of a certain size, begin to deposit yolk. Representative stages of these processes are shown in Figures 2, 3, and 4.

It must be emphasized concerning this response that not all the oöcytes in a given ovary pass through the above stages simultaneously; some may appear to be not at all affected, and in other cases stages of initial yolk resorption are seen in the same section with numerous, newly formed oöcytes. Indeed, all indications are that the difference in size be-

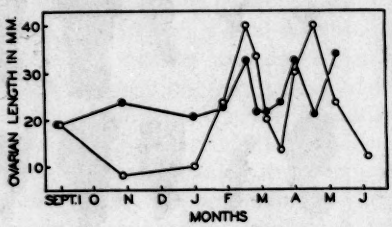

FIG. 15.—Relation of ovarian length in millimeters for 20-hour and normal-day-length animals, to time in months. Twenty-hour: ○; normal-day-length: ●.

tween the 20-hour and normal-day-length specimens of Figure 13 is based principally on relative numbers of oöcytes affected simultaneously and that, once yolk begins to be withdrawn from a given oöcyte, the destruction process goes on to completion. Thus the process is not just one of a mobilization of yolk into and out of the same population of öocytes.

C. CONCLUSIONS AND DISCUSSION

An analysis of the above three groups of data permit several conclusions.

1. Animals exposed to daily light periods during the months from September through early June tend to undergo a periodic resorption of oöcyte yolk (which process, if extensive enough, results in a coloration of the blood with these substances) and subsequent destruction of

the oöcyte as a whole. This phase alternates with a period of oöcyte formation and maturation. The initial responses to the light conditions cover a 4-month period, in which the ovaries of the 20-hour animals undergo a protracted resorptive phase, while those of the normal-day-length group go through a period of further maturation, later followed by a resorptive phase. Once the more rapid cyclical activity is initiated, it is not interrupted either by egg-laying, at the one extreme, or by a period of quiescence of the immature oöcytes, at the other.

2. The duration of the photoperiod which is administered apparently does not influence the frequency of the cycle in that portion of the population responding by resorption of yolk extensive enough to color the blood, since rises and falls in percentages occur at approximately the same periods in both normal and 20-hour day-lengths. The exception to this statement is the apparently earlier onset of the third resorptive phase in the normal-day-length group; it is possible that this response is occasioned by a photoperiod which is in the process of increasing above a critical threshold value. Whatever the mechanism, it apparently involves something more than just total daily light ration, since the photoperiod is well below 20 hours and all animals are responding.

3. Duration of the photoperiod does, however, influence the amplitude of the cycle as concerns the number of oöcytes affected simultaneously in a given animal and the number of animals affected in a given group. Apparently, there are varying thresholds of sensitivity to light among the animals making up a population; the longer the duration of the photoperiod administered, the greater is the number of animals influenced to a degree adequate for causing blood-color changes. Here again the factor of an increasing photoperiod may be significant in the normal-day-length group, where in early June all the animals were responding in this way to a light duration significantly lower than 20 hours.

4. Twenty-hour light appears to act so as to synchronize the responses of the entire group quite closely, as may be seen by comparing Figures 14 and 15, where one curve is, in a sense, the inverse of the other. Although the number of animals showing blood-color shifts in the resorptive phase of late March is significantly lower than that in the phases of October and June, the ovaries of the group returned to a near-basal state. An inherent, perhaps annual, cycle of susceptibility to environmental conditions may explain this observation.

5. Normal day-length serves to correlate the responses of the group to a lesser extent than 20-hour light periods. While a small number of animals responded to the light durations of October by resorption, the majority underwent further maturation and hence increase in ovarian size. From February through early April there is some degree of correlation, but thereafter the oöcyte cycle accelerates, without significantly increasing its amplitude, until June, when, as mentioned before, the animals respond synchronously. If these animals could have been autopsied in late July, there is not a question but that the ovaries would all have been found to be in the basal condition.

6. Under conditions of continuous darkness the majority of animals undergo progressive oöcyte maturation and consequent increase in size of the ovary until an apparent maximum is attained in February.

III. Destalking Experiments

Since it has been demonstrated that the sinus gland exerted an inhibitory ef-

fect upon the early maturation phase of the ovarian cycle which occurs in June, July, and August, it became of interest to determine the effect of eyestalk removal during the fall and early winter, when the ovary contains the large brown-green oöcytes mentioned in the previous section. It had been assumed previously that oöcyte maturation was completed by late summer or early fall and then that the eggs were simply stored within the ovary until spring, when egg-laying (in *C. virilis*) occurred at a variable period of time, depending on the weather, following emergence from hibernation.

A. RESULTS

Groups of females, approximately twenty for each experiment, had their eyestalks removed at intervals, as animal shipments arrived in the laboratory, and were examined on successive days following the operation to observe its results. It was found that eyestalk removal during the period covered by the experiments (early September through January) may have several possible effects. As might be expected, when the animals survive for only short periods of time following the operation, there are no changes observable at autopsy.

1. A slight increase in ovarian size over that of the controls may be seen, probably indicating accelerated yolk deposition. This response was observed only in animals of the September group, and only in those of this group which did not live more than about 3 weeks.

2. The animals may lay eggs. These results are presented graphically in Figure 16, where cumulative percentages of animals having laid eggs in each of the experiments is plotted against days following eyestalk removal. None of the control animals laid eggs in any of these experiments. Average temperatures in the tanks for the time covered by each experiment are shown.

Ovaries of destalked animals which had laid eggs were studied histologically. A month after eyestalk removal, when egg-laying occurred on the day following the operation, the appearance of the ovary is quite similar to that of the normal an equal number of days following deposition of eggs. (It should be pointed out that the destalked animal was from

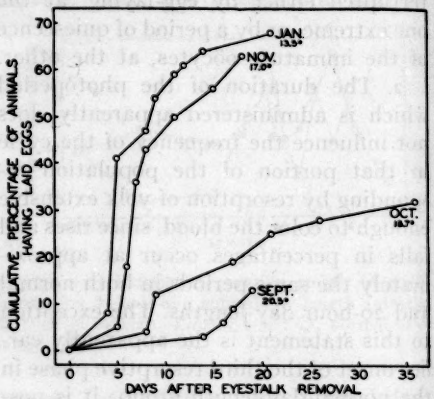

FIG. 16.—Graphic representation of the relation between percentage of animals having laid eggs, and days after eyestalk removal, for the months indicated. Average water temperatures for the period covered by each experiment are shown.

the January group and the normal animal arrived in the laboratory the end of February.) An experimental ovary is shown in Figure 12. In the few animals which survived the operation for 3 months after eyestalk removal and egg-laying, the ovary is seen to contain a significantly smaller number of young and developing oöcytes than the 30-day animals; there is no yolk deposition occurring (contrast with the cycle of normal animals exposed to light); and there is marked aplasia of the ovarian stroma (Fig. 5).

3. Resorption of yolk from the oöcytes may occur (*a*) in animals of the Septem-

ber group living for about a month or more after eyestalk removal and (*b*) in most of the animals of the other experiments which did not lay eggs. As is the case with egg-laying, the latent period between eyestalk removal and response decreases markedly with time in each of the experiments. Because of this time decrease and since the viability of the animals is generally limited after eyestalk removal, the percentage of animals showing active resorption increased in October. It then dropped off progressively in November and January as an increasing number responded by egg-laying.

As discussed under the light experiments, if yolk resorption is extensive enough—and in eyestalk removal it generally is—substances mobilized as a result of this process are liberated into the blood stream so rapidly that the blood is colored orange or red. Gross examination of the ovary reveals the variegated condition previously described; but in animals which survived for long periods (much over a month) and whose ovaries were studied histologically, the condition is seen to be quite different from that of normal animals undergoing the oöcyte cycle described in the light experiments. The whole interior of the ovary is seen to be a disorganized mass of homogeneous yolk material; the epithelial boundaries of these centrally lying oöcytes have degenerated, and their place is taken by a substance which resembles yolk but takes a somewhat lighter stain with acid fuchsin. A few "wandering" cells can be seen. Only at the extreme periphery of the ovary does the process of oöcyte destruction resemble that described in the light experiments, but, in addition, there is profuse yolk-vacuole formation in the oöcyte epithelium. Here, however, a cycle is never completed. At the periphery, when a few new oöcytes form, they do not grow to any appreciable size, and yolk deposition does not occur. The deteriorated condition of the central mass of the ovary persists, and the animal eventually dies. This situation, typical of destalked animals surviving for 40 days, is shown in Figure 6.

It should be emphasized here for future reference that, in a few cases in this group of destalking experiments, animals which have started to resorb their oöcytes may subsequently lay eggs and that in many cases egg-laying is incomplete and resorption of unlaid eggs occurs extensively enough to color the blood. Thus these two processes—yolk resorption and egg-laying—are not at all mutually exclusive, but their controlling mechanisms appear to be very closely related.

B. CONCLUSIONS

From this series of experiments dealing with the results of eyestalk removal, one may make the following conclusions: (1) The tendency to respond to destalking by the laying of eggs increases gradually during the winter, implying that processes of oöcyte maturation are proceeding under natural conditions of cold and darkness. The latent period for this response decreases during this period. (2) In animals whose oöcytes have not yet attained the degree of maturity permitting egg-laying, the tendency to respond to destalking by increased yolk deposition drops off rather sharply, to be succeeded by (3) a tendency to resorption of the already deposited yolk, a response which, like egg-laying, shows a latent period which decreases with increasing maturity of the oöcytes. (4) The presence of the sinus gland may be essential for (*a*) yolk deposition in newly formed oöcytes; (*b*) a complete and normal process of yolk withdrawal from partially matured oöcytes, although *its presence inhibits such withdrawal* (at first, this

may appear to be a paradoxical statement, but resorption of yolk is one of the responses induced by eyestalk removal; yet the process is never completed as it is in the normal animal); (*c*) continued oöcyte maturation after approximately the end of September; and (*d*) general maintenance of the ovary.

IV. Implant Experiments

A. Materials and methods

These experiments were undertaken with the idea of attempting to elucidate the factor, possibly within the central nervous system, which is responsible for the stimulation of egg-laying and/or resorption of yolk from the oöcytes. The tissues being tested were removed from donor animals by dissection, with the aid of a dissecting microscope when necessary, drawn by means of a tuberculin syringe into the lumen of a 16-gauge needle, and injected with 0.25 cc. of Van Harraveld's solution into the ventral hemocoel of the recipients through the transparent chitinous area of the ventral abdomen. The process of extrusion of the implant is readily observable through this clear area. In order to assess the viability of these implants, central nervous tissues which had been in place for 30 days were removed, stained with methylene blue, and compared under the microscope with freshly removed nervous tissue which had been similarly stained. The two specimens were similar in appearance; of course, this is only presumptive evidence for a continuance of functional integrity.

B. Results

It may be stated at the outset that the slight increase in percentages of animals laying eggs sometimes obtained in the implanted group over that of the controls (both groups with their eyestalks removed) was not considered great enough to be significant. Also, since it was necessary to employ male animals as the donors of central nervous tissues, owing to a dearth of females available, it was thought unlikely that acceleration of the process of yolk deposition would occur. This, indeed, turned out to be the case, since no such effect was observed.

At approximately weekly intervals from September 2 to October 7 three groups, of thirty normal animals each, received the following types of implants, respectively, according to the above-outlined techniques: group 1, cerebral ganglia and circumesophageal connectives; group 2, a lateral half of thoracic cord (approximately equal in volume of tissue to one "brain" and set of connectives); group 3, an equal volume of macerated abdominal muscle. On October 9 all the animals had their eyestalks removed, and 10 days later five animals from each group were sacrificed. At this time six of the "brain"-and-connectives animals were showing red blood color, while none of the animals of the other two groups was doing so. Though the animals from the "brain"-connective group chosen for sacrificing still had the normal clear blood, their oöcytes were demonstrating initial stages of yolk resorption; again, this was not seen in the sacrificed specimens of the other two groups. These ovaries were preserved and examined histologically. Figure 7 shows a section of ovary removed from one of the "brain"-connective implanted animals, and Figure 8 an ovary from an animal receiving thoracic cord implants. (In Fig. 8 the oöcytes have attained a degree of maturity which makes sectioning difficult; the yolk-laden cytoplasm hardens and crumbles.) The process of yolk resorption and even new oöcyte formation are evident in the former, while the latter resembles the normal in every respect.

A similar experiment was performed in late October with animals whose eyestalks had been ablated a day before the first implant was given. Here the differences in time of onset and degree of yolk resorption among the groups were less marked, as might be expected from recalling the results of the experiments discussed in the section "Destalking Experiments," where it was shown that, from September through January, animals tend to respond more readily to eyestalk removal by the resorption of yolk.

In early January another such implant experiment was performed. Thirty eyestalkless animals received two ganglia and connectives implants per week for 3 weeks, while the control group received muscle implants at the same frequency. Again, degree of egg-laying was about the same in the two groups, as was time of onset and degree of blood-color change. In this case, however, ovaries of animals of both groups which had laid eggs were preserved and examined histologically. Figures 9 and 10 are from two experimental animals which both laid eggs on the second day following eyestalk removal and which were sacrificed 8 and 35 days, respectively, after egg-laying. Figures 11 and 12 are from controls which were destalked, laid eggs, and were sacrificed at the same times as the experimentals. It is of interest to observe the much greater numbers of new oöcytes formed in the control animals, and the extreme proliferation of non-oöcyte material in the ones implanted with cerebral ganglia and connectives.

C. CONCLUSIONS

The results of these experiments might lead one to think in terms of the following possibilities:

1. Implants of cerebral ganglia and connectives from male donors into eyestalkless females in early fall seem to bring about resorption of the yolk, which has been deposited in the maturing oöcyte, more rapidly than destalking itself will do.

2. The change in reactivity of the female as winter progresses is such that eyestalk removal alone eventually has as great an effect as do these implants.

3. Implants of thoracic cord from male donors, in the quantities used in these experiments, appear not to have the effect mentioned in paragraph 1.

4. There may be some factor in the central nervous tissues of male animals which, when implanted in the female, (*a*) causes cellular proliferation in the ovary and (*b*) acts so as to inhibit the formation of new oöcytes.

V. Discussion and a Theory of the Mechanisms Responsible for Cyclical Reproductive Activity in the Female

On the basis of the relevant facts cited in the introduction and the results of this series of experiments, it is possible to construct a hypothesis of the regulatory factors, environmental and organismic, which are responsible for the reproductive cycle in the female crayfish. Several of the statements which will be made are as yet untested but would lend themselves readily to experimental investigations, some of which are now in progress.

Let us assume the following:

I. The sinus gland secretes two substances associated with reproduction in the female.
   - A. An inhibitor of ovarian activity in all phases of the cycle. This concept is based on experimental results from the present work and from the work of Brown and Jones (1947) cited previously.
   - B. A substance which is used by neurosecretory cells of the brain

to conjugate the hormone which, in the active state, produces concentration of the pigment of the white chromatophores; let us call this sinus gland substance "S." This substance is postulated, since it seems evident that the sinus gland is related in an intimate fashion to the reproductive cycle of the female. That this relationship is something more than the mere inhibition of ovarian processes proceeding under the totally autonomous regulation of some organ other than the sinus gland is indicated by the fact that, during the period of yolk deposition, at one time the rate of the process is changed by the operation and at another an entirely different process is instigated.

The postulated characteristics of the sinus gland's secretion of S are based on the results of the light experiments and will be related to these in greater detail shortly. They are as follows:

1. Light inhibits the secretion of S.
2. The sinus gland eventually becomes refractory to inhibition and can spontaneously recover from inhibition under continued light.
3. If the light exposure continues beyond a certain critical period, the sinus gland again becomes inhibitable, and secretion of S ceases.
4. Darkness promotes the secretion of S.

II. The "brain" secretes two substances associated with reproduction in the female:

A. A substance with the following functions or activities:

1. It concentrates the pigment of the white chromatophores; call it "WC-active."
2. It induces proliferation of the interstitial tissue and, in optimal concentrations in normal animals, the formation of new oöcytes in either freshly evacuated ovaries or ones actively resorbing yolk.
3. It causes egg-laying of oöcytes which have reached the appropriate degree of maturity.
4. It induces resorption of yolk and destruction of oöcytes, with accompanying proliferation of the follicular epithelium, under certain physiological states of the ovary, such as relative immaturity.
5. It is stimulated to form in the "brain" by light.

B. A substance which we shall call "WC-conj." and which

1. Is A, above, in the conjugated form and, until boiled, does not concentrate the white chromatophores.
2. Is formed in the "brain" as the result of the secretion on the part of the sinus gland of the substance S.
3. Causes and regulates deposition of yolk in the oöcytes.

One basis for these postulated relationships of the cerebral ganglia to the reproductive cycle of the female is the work of McVay (1942), described previously. Now, as we have seen in the present work, normal animals during the fall and winter months are undergoing progressive oöcyte maturation; thus this process is correlated with a certain secretory state of the cerebral ganglia. Also, egg-laying and/or resorption of yolk from the oöcytes and formation of new oöcytes are three processes seen to occur concur-

rently or in close sequence under both normal and experimental conditions. These processes are thus correlated with the alternative secretory condition of cells of the "brain."

As is the case with the sinus gland, the relationship between "brain" and light exposure will be discussed fully in succeeding paragraphs.

In terms of the assumptions just enumerated, it is possible to discuss the normal yearly cycle of the female as follows: In early spring the oöcytes have undergone the complete maturation process; warm weather comes, and the animals emerge from their burrows, as a result of which they are exposed to light. The fact that the photoperiod is increasing may be of significance. The action of this light is (*a*) to prevent the sinus gland from secreting S and (*b*) to stimulate the brain directly to increase its secretion of WC-active. When the blood titer rises sufficiently, the WC-active induces the changes immediately preparatory to egg-laying, which then occurs. At this same time there is proliferation of the germinal epithelium under continued light stimulation, and new oöcytes form very shortly after evacuation of the ovary.

In late June the sinus gland becomes refractory to further inhibition by light and begins secreting S. As a result of the presence of the brain hormone in the conjugated form, yolk deposition begins. This initial stimulus to the brain is a powerful one, and yolk deposition is at first autonomously regulated by the brain, so that in this process, for the next 2–3 months, the sinus gland is acting principally in its role of general inhibitor of ovarian activity. Ovarian maturation proceeds gradually, but, as this maturation goes on, the brain becomes less and less capable of independently conjugating WC-active. We can possibly assume here that quantities of S are stored by these neurosecretory cells, the supply gradually being depleted and thus eventually, since during this time the sinus gland continues secreting S, the brain comes again to rely on the sinus gland for S.

In forms which deposit their eggs in the fall, such as *C. immunis* of certain regions (Tack, 1941), it is likely that ovarian maturation has proceeded more rapidly (earlier start because of climatic differences; the sinus gland has not exerted so strong an inhibitory influence; etc.). The sinus gland recovers from refractoriness and is once again inhibitable. Thus the brain secretes WC-active, and egg-laying occurs. If hibernation then takes place, the cycle is temporarily stopped, since continued light is necessary for new oöcyte formation; if it does not occur, oöcytes form and yolk deposition begins when the sinus gland recovers its capacity to secrete S.

In forms which, like the animals of these experiments, do not lay eggs in the fall but mature their oöcytes over the winter and lay in the spring, the situation may be as follows. Day-length in the fall of the year has decreased considerably; because of this drop-off, the reinhibition of the sinus gland would probably be slower to take effect, so that the gland has a much greater tendency to protract the secretion of S than if this phase of the cycle were pushed back to mid- or early summer. Even with these short photoperiods, the sinus gland would become inhibitable eventually. However, by the time this could occur, cold weather would have stimulated the animals to seek hibernacula and consequent darkness. So the gland continues secreting S, and yolk deposition and consequent ovarian maturation continue throughout the winter.

Under experimental conditions of continued long and uniform photoperiods,

such a cycle is compressed and altered thus: animals which in the fall are undergoing normal yolk deposition are placed in a 20-hour light environment. The sinus gland is inhibited from secreting S, and the "brain" is stimulated to secrete even greater amounts of WC, which now cannot be conjugated. Yolk is mobilized from the oöcytes, though this rate of mobilization is somewhat retarded by the sinus gland acting as general inhibitor. The partially matured oöcytes are destroyed; as new oöcytes form and reach the stage appropriate for the initiation of yolk deposition, the gland recovers from inhibition, secretes large quantities of S, yolk deposition ensues, and the oöcytes greatly increase in size. The gland again becomes inhibitable, S secretion stops, and the whole cycle is repeated again and again. Egg-laying never ensues because the sinus gland is inhibited before maturation of the oöcytes is complete.

The situation in the normal-day-length experiments must be somewhat different. As we have seen in the majority of animals, the extremes of size are never reached in light periods of short duration, as they are in the 20-hour group. With the increase in light duration in the spring, however, the situation comes to resemble more closely that of the 20-hour animals, with complete substitution of a new set of oöcytes for the old. Also, it will be recalled that the majority of animals did not undergo oöcyte resorption upon being placed under exposure to normal day-lengths of September and October but continued to mature their oöcytes; the sinus gland was therefore not inhibited from secreting S until later, in December, when the majority of the group show reduction in size of the ovary. The acceleration of the oöcyte cycle seen in the normal-day-length group from March 1 on may be explained by the fact that, as the photoperiod increases, the sinus gland requires less time both to recover spontaneously from light inhibition and to become re-inhibited. There are probably threshold values for each response. In addition, one may be sure that the responses occurring in each phase of the cycle in both normal-day-length and 20-hour animals are importantly influenced by, and must be explained partially in terms of, what has occurred in previous stages of the cycle.

VI. Summary

1. When female crayfishes are exposed to photoperiods of varying durations from September through the first of June, the ovaries undergo processes of yolk resorption and oöcyte destruction, alternating with a formation and maturation of these structures which does not culminate in egg-laying. The events occur in a cyclical fashion.

2. The phase of yolk resorption is frequently accompanied by mobilization of yolk substances to such a degree that the blood is changed from its normal clear state to a deep orange or red color.

3. Certain important characteristics of this cycle are different under conditions of 20-hour light and normal day-length. In the former case, the oöcyte and blood-coloration cycles are closely correlated in the whole population. In normal day-length a small percentage of the animals show a response which corresponds to the 20-hour curve. However, the majority respond in a quite different fashion, which appears to be correlated both with the absolute length of the photoperiod and with its gradually changing character, that is, with its increase or decrease, as the case may be.

4. Under conditions of complete darkness, no cyclical activity is seen; the oöcytes undergo progressive maturation.

5. The tendency to respond to eyestalk removal by the laying of eggs in-

creases markedly from September through January.

6. The tendency to respond to eyestalk removal by increased yolk deposition drops off rather sharply in late September, to be succeeded by a tendency to respond to this removal by extensive resorption of already deposited oöcyte yolk.

7. The presence of the eyestalk is essential for the complete process of yolk withdrawal from partially matured oöcytes, exemplified in the light experiments; yet eyestalk removal induces an initiation of yolk resorption.

8. There appears to be some substance in certain nervous tissues of the male crayfish which, when implanted in the female, promotes yolk resorption from partially matured oöcytes and, after egg-laying, (*a*) causes proliferation of interstitial material in the ovary and (*b*) inhibits the formation of new oöcytes.

9. The experiments have revealed little as to the factors involved in egg-laying.

10. A theory of the mechanisms regulating the reproductive cycle in the female crayfish is presented.

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PLATE I

FIG. 1.—Section of ovarian tissue from a normal animal; typical of stage of maturity in early September. ×66.

FIG. 2.—Initial stages of yolk resorption from oöcytes under conditions of 20-hour photoperiods. ×66.

FIG. 3.—Stages of destruction of oöcyte cytoplasm under conditions of 20-hour photoperiods. ×66.

FIG. 4.—Late stages of oöcyte destruction and formation of new oöcytes in 20-hour photoperiods. ×66.

FIG. 5.—Ovarian tissue from an animal which had laid eggs the day following eyestalk removal, 90 days previous to autopsy. ×66.

FIG. 6.—Ovarian tissue from an animal which had been destalked 40 days previous to autopsy; animal had not laid eggs. ×66.

PLATE II

FIG. 7.—Ovarian tissue from an animal which had received a series of implants of "brain" and connectives before and after eyestalk removal and which was sacrificed 10 days after the operation. ×66.

FIG. 8.—Ovarian tissue from an animal which had received a series of implants of thoracic cord before and after eyestalk removal and which was sacrificed 10 days after this operation. ×66.

FIG. 9.—Ovarian tissue from an animal which had been destalked and then received a series of implants of "brain" and connectives; sacrificed 8 days after egg-laying. ×66.

FIG. 10.—Ovarian tissue from an animal which had been destalked and then received a series of implants of "brain" and connectives; sacrificed 35 days after egg-laying. ×66.

FIG. 11.—Ovarian tissue from an animal which had been destalked and was sacrificed 8 days after egg-laying. Control to the animal of Fig. 9. ×66.

FIG. 12.—Ovarian tissue from an animal which had been destalked and was sacrificed 35 days after egg-laying. Control to the animal of Fig. 10. ×66.

ADAPTIVE RESPONSES TO NEUROHYPOPHYSEAL FRACTIONS IN VERTEBRATES[1]

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THE pharmacological effects of posterior pituitary extracts on mammals have been subject to extensive investigation in the half-century since their discovery. Of these effects, only the tubular antidiuretic effect on water excretion has been generally accepted as of physiological significance. The vasopressor and oxytocic effects, although of pharmacological interest, have not been proved to play important roles in physiological regulation.

Posterior pituitary extracts may be chemically separated into two fractions —oxytocic and vasopressor. The oxytocic fraction contains the principle that produces contraction of the isolated uterus. The unit of oxytocic activity is defined as the oxytocic potency of 0.5 mg. of the international standard posterior pituitary powder. The vasopressor fraction is characterized by its ability to elevate the blood pressure of anesthetized or decerebrate mammals. One pressor unit is that contained in 0.5 mg. of the standard powder. The tubular antidiuretic principle is apparently identical or closely related to the vasopressor principle. Both oxytocic and vasopressor activities are present in whole posterior pituitary extract, usually in a ratio of 1 oxytocic unit to 1 pressor unit.

The true nature of the neurohypophyseal secretion is a matter of debate. There is little evidence that the fractions exist as such in the body, and it appears

[1] Aided in part by a grant from the William F. Milton Fund.

PLATE I

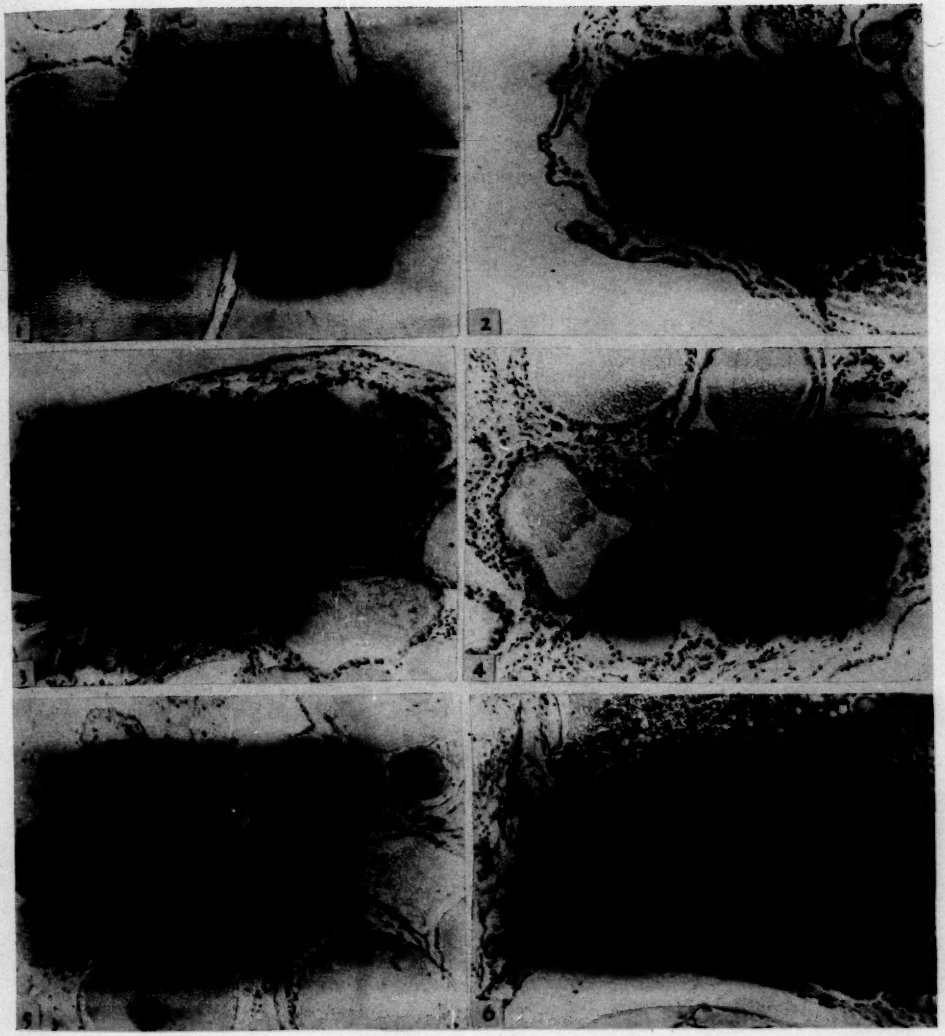
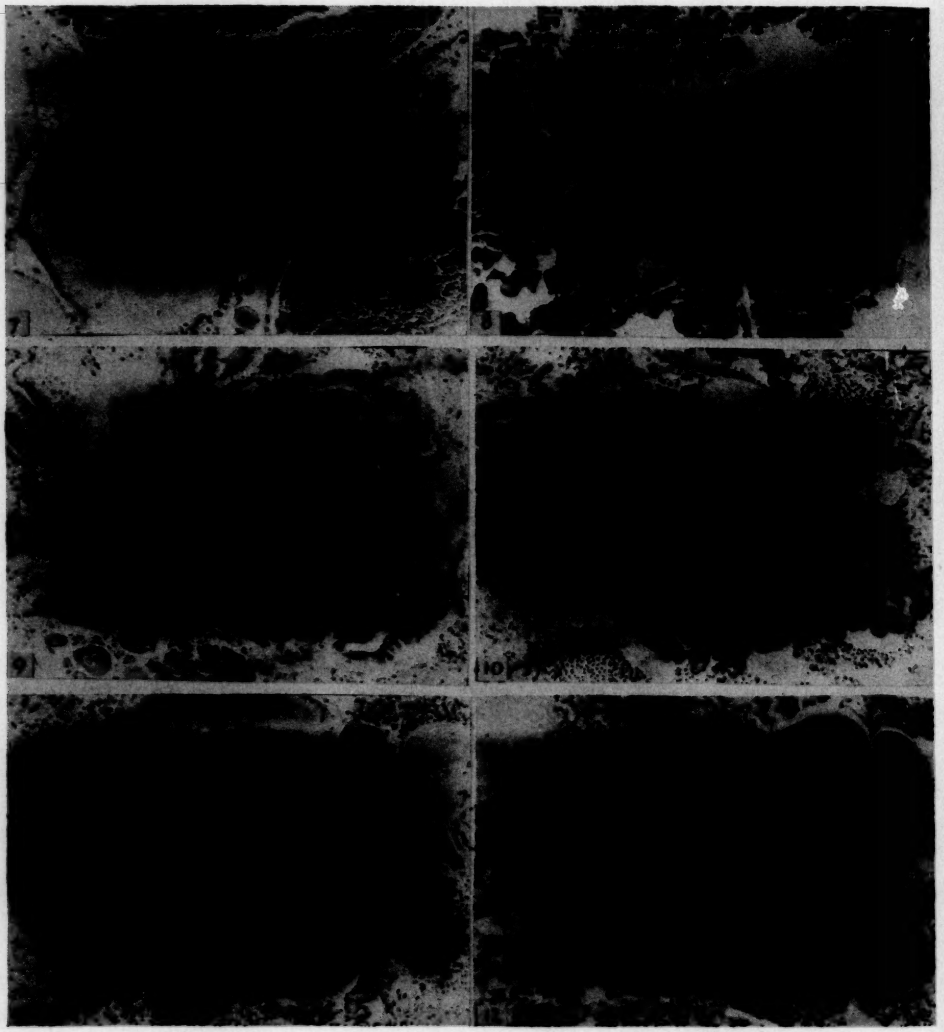


PLATE II



unlikely that either can be secreted alone. The existence of a single large protein molecule containing oxytocic and pressor activities in a fixed ratio has been demonstrated in the ox pituitary by Van Dyke *et al.* (1941). It is possible that this protein may be secreted as the true hormone and later hydrolyzed, or it may remain intact under physiological conditions. Whether the fractions represent artificial fragments of this parent-hormone or separate hormones, they are extremely useful in the investigation of the specific properties that each may possess.

Responses to neurohypophyseal extracts among vertebrates other than mammals have been studied rather thoroughly, but the results have often appeared conflicting. Several responses differing from those in mammals have been described. Paton and Watson (1912) found that posterior pituitary extract exerted a vasodepressor effect, causing a fall in blood pressure, in birds. Brunn (1921) observed that posterior pituitary extract could produce an increase in the rate of water uptake through the skin of the frog; this has been called the "water-balance" response. Burgess, Harvey, and Marshall (1933) described the glomerular antidiuretic response of reptiles and birds, a decrease in glomerular filtration and urine formation after administration of posterior pituitary extracts.

Investigation of the phyletic distribution and character of these responses may help in understanding their importance in mammalian physiology. It also provides an interesting picture of a series of adaptations of responses to the product of an endocrine gland occurring during the course of vertebrate evolution. Study of the fractions possessing the various activities affecting different animals may shed some light on the problem of the relative importance of these fractions as opposed to that of the parent-hormone.

Heller (1945, 1950) has reviewed thoroughly the literature on the comparative physiology of the neurohypophysis, and detailed review here would be superfluous.

MATERIAL AND METHODS

I. MATERIALS

Rana pipiens were obtained from a Vermont supplier. Males, weighing about 30 gm., were used. *Rena catesbiana* of both sexes, weighing 350–650 gm., were shipped from Louisiana. The frogs were kept in tanks of running water and were not fed. The bullfrogs showed signs of early "red leg" (*Pseudomonas hydrophila* septicemia) when received. Wood (1951) has shown that very small doses of chloromycetin (chloramphenicol) will cure this disease in *R. pipiens*. The bullfrogs were given two daily doses of 10 mg/kg intraperitoneally. This treatment was sufficient to prevent the advance of the disease, and reinfection was carefully avoided.

Twelve *Bufo marinus* of both sexes, weighing 360–775 gm., were shipped by air from Bermuda. They were kept in a moist terrarium and fed chopped horse meat weekly. Six young alligators, between two and three feet in length, weighing 445–765 gm., were obtained from Louisiana. They were kept in a tank at 27° C. and fed chopped horse meat. During a 10-month period they increased in length and doubled in weight.

II. METHODS

a) Production of diuresis.—*Rana pipiens* were given an injection of 0.05 N NaCl, 100 ml/kg, into the dorsal lymph sac. *Rana catesbiana* and *B. marinus* received 50 ml/kg water by injection. Alligators were given a similar volume of water by stomach tube.

b) Administration of extracts.—Pituitrin (posterior pituitary solution, U.S.P.),

pitocin (N.N.R.), and pitressin (N.N.R.), all Parke, Davis products, were used in these experiments. These preparations have been used so widely that their trade-names have become synonymous with whole posterior pituitary extract, oxytocic extract, and vasopressor extract, respectively. It is common practice to use them thus, and, for the sake of convenience, that will be done here.

In all the experiments the volume of injections were kept constant among the various groups. Control animals always received injections of chloretone solution in volume and concentration equal to that present as a preservative in the pituitary solutions. All injections were subcutaneous except in the blood-pressure experiments. In these the extracts were administered through a fine polyethylene cannula tied into a vein. Control injections of Ringer's and chloretone were without significant effect on blood pressure.

In the experiments on bullfrogs, toads, and alligators in which two or more extracts were to be compared, the design was such that each animal received each treatment, but not in the same order. Thus each individual served as a control on itself as well as on the others in the group. In the experiments on *R. pipiens* a simple crossover design was employed.

c) Administration of inulin and creatinine.—Bufo marinus received 250 mg. of creatinine and 250 mg/kg of inulin in the initial water load given for the clearance experiments. The first clearance period was started 1 hour later.

A solution of creatinine and inulin, 2 per cent of each, in physiological saline was injected intraperitoneally in a dose of 5 ml/kg into the alligators 1 hour before the initial clearance period.

d) Collection of urine and blood.—Rana pipiens were placed in covered glass funnels for 1 hour following injections. At the end of this period the urine was expressed by manual pressure into the funnel and collected in graduated centrifuge tubes. Bullfrogs were kept in large jars containing 500 ml. tap water. They were catheterized at hourly intervals. *Bufo marinus* were kept in individual rat metabolism cages. They were catheterized with a special glass cannula every half-hour. Despite precautions, many of the urine samples were heavily contaminated with feces, and filtering failed to remove all the color present, which interfered somewhat with colorimetric determinations.

Midpoint blood samples were drawn from the heart for each clearance period. Between 0.3 and 0.5 ml. of blood was transferred to a small oxalate tube and centrifuged. The plasma was deproteinized with cadmium sulphate and sodium hydroxide after suitable dilution. The protein-free filtrate was analyzed for inulin by a modification of Harrison's (1942) method and for creatinine by the alkaline picrate method (Peters and Van Slyke, 1932). Diluted urines were analyzed simultaneously by the same methods. Urine chloride was determined by a modified Volhard technique (Peters and Van Slyke, 1932).

e) Blood-pressure determinations.—Bullfrogs were anesthetized with nembutal (sodium pentobarbital) or were pithed. After pithing, bone wax was injected into the cranial cavity and the upper spinal canal to reduce blood loss. *Bufo marinus* were anesthetized with urethane or were pithed in a similar manner. The responses of frogs and toads were essentially the same under anesthesia as after pithing. The alligators were anesthetized with nembutal.

Artificial respiration was administered by means of a pump and a catheter inserted through the glottis of the toad and frog or by tracheal cannulation in the

alligator. Fine polyethylene tubing was tied into the carotid artery in the toad and the alligator and into the subclavian artery in the frog. This was connected to a recording mercury manometer by means of tubing containing citrate solution. A second polyethylene tube was tied into the jugular or the ventral abdominal vein. Injection into either vein appeared to cause an equivalent effect.

Following each injection of test extract, the tubing was flushed with a constant volume of Ringer's solution.

RESULTS

1. FROGS

It has been shown that the oxytocic fraction exerts both antidiuretic and water-balance activity in *R. pipiens* (Sawyer *et al.*, 1950; Sawyer, 1951*b*). The antidiuretic effect appears to be of the glomerular type, causing constriction of the afferent glomerular arteriole and a reduction in filtration rate. In this respect it resembles the response observed in the alligator (Burgess *et al.*, 1933). There is little, however, known about the existence or identity of a chloruretic principle causing increased excretion of chloride in amphibians. Boyd and Whyte (1939) reported that pitressin and pituitrin hasten the excretion of injected saline solution by *R. pipiens*, but Oldham (1936) and Sawyer (1951*b*) observed that pituitrin could inhibit a diuresis caused by the administration of physiological saline solutions.

Doses of posterior pituitary extracts that are not antidiuretic were administered to *R. pipiens* simultaneously with 100 ml/kg of dilute saline. There was a consistent increase in urine chloride concentration and in absolute chloride excretion in the following hour (Table 1). This chloruretic effect seems to reside in the oxytocic fraction rather than in the vasopressor fraction. In this respect the frog resembles the rat (Dicker and Heller, 1946; Sawyer, 1951*c*).

TABLE 1

EFFECTS OF POSTERIOR PITUITARY EXTRACTS ON CHLORIDE EXCRETION BY *Rana pipiens* IN 1 HOUR FOLLOWING INJECTION OF 100 ML/KG 0.05 N NaCl

Extract	No. of Animals	Dose (U/Kg)	Urinary Chloride	
			Concentration (MEq/L)	Total amount (MEq/Kg)
Controls	32	0.0	10.9±1.35	0.297
Pituitrin	16	1.0	20.0±1.78	0.403
Pituitrin	8	0.5	19.5±2.49	0.357
Pituitrin	8	0.2	10.8±3.25	0.174
Pitocin	11	0.5	19.9±2.54	0.339
Pitressin	15	0.5	15.0±2.37	0.297

Burgess, Harvey, and Marshall (1933) were unable to detect an antidiuretic effect of pitressin or pituitrin on *R. catesbiana*, although they used doses as high as 2 units/kg. Since this dose produces a slight response, at best, in *R. pipiens* (Sawyer, 1951*b*), it appeared possible that they had stopped just short of an effective dose. When 1 and 10 units/kg were administered to six bullfrogs, a definite antidiuretic effect was observed (Fig. 1). The response appears in the

same dose range as that in *R. pipiens*. When pitressin and pitocin are given in comparable doses of 10 units/kg, it appears that pitocin is essentially as effective as pituitrin (Fig. 2). Ten units of pitressin have less antidiuretic effect

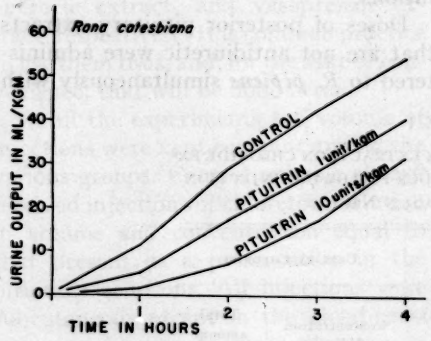


FIG. 1.—The effect of pituitrin on water excretion by the bullfrog.

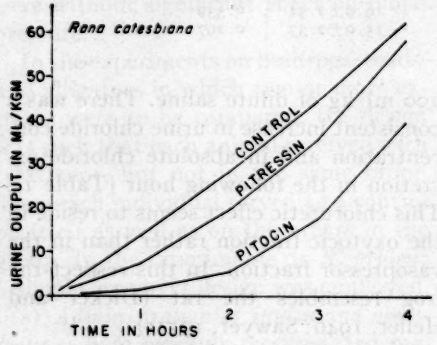


FIG. 2.—The effects of pitocin and pitressin, 10 units/kg, on water excretion by the bullfrog.

than does 1 unit of pituitrin. This confirms the findings in *R. pipiens* that the oxytocic fraction is antidiuretic to frogs. It would also appear likely that the antidiuretic effect of pitressin is due to its content of oxytocic principle. The pitressin used for these experiments was found to contain 4 per cent of the oxytocic potency of pitocin when compared by the rat uterus assay method of Holton (1948).

It had previously been observed that relatively large doses of pitressin administered intravascularly had little effect on peripheral blood flow in *R. pipiens* (Sawyer, 1951*b*). In *R. catesbiana* it was possible to record the changes in systemic blood pressure produced by the intravenous injection of posterior pituitary extracts (Fig. 3). The administration of either pitocin or pituitrin almost invariably provokes a fall in blood pressure (Table 2). This vasodepressor effect is

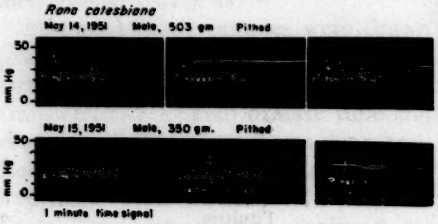


FIG. 3.—The effects of neurohypophyseal extracts on blood pressure in the bullfrog.

destroyed by brief boiling in 0.1 N NaOH and is thus not due to histamine contamination (Hogben and Schlapp, 1924). Pitressin itself, however, is strangely ineffective in producing any marked rise in blood pressure. Relatively high doses do not always evoke a rise and frequently cause a definite depression, probably due to the presence of oxytocic activity. Occasionally a diphasic response, consisting of a fall followed by a rise, is observed after pitressin administration. It appears that the frog resembles the bird, in that pitocin consistently causes a fall in blood pressure (Coon, 1939), but is not so sensitive. The pressor response to pitressin is much weaker and slower than that found in either birds or mammals. The depressor effect becomes less marked with repeated injections. The pressor response is too variable to allow determina-

tion of the development of resistance with time.

Since pitressin is extracted from mammalian hypophyses, it was felt that the lack of pressor effect on the frog might be due to the fact that this was a foreign hormone. An extract of *R. catesbiana* pituitaries was prepared by boiling homogenized glands in 0.25 per cent acetic acid in Ringer's solution. The intravenous administration of this extract to a pithed bullfrog in a dose corresponding to ½ pituitary/kg caused but a small decrease in blood pressure, somewhat less than that observed after one unit of pitocin (Fig. 3). No pressor effect could be elicited. Brief boiling in 0.1 N NaOH destroyed the vasodepressor effect of this extract, indicating that it was not due to histamine. Hogben and Schlapp (1924) found that similar treatment destroyed the vasodepressor activity of whole pituitary extracts on birds and turtles and concluded that it was a specific property of posterior pituitary substances.

The bullfrog pituitary extract was assayed by the isolated rat uterus method of Holton (1948). It was found to contain 0.35 oxytocic unit per pituitary. Although the rat uterus is extremely insensitive to histamine (Sawyer, 1951*c*), alkali deactivation was tested. This completely eliminated all oxytocic activity of the extract. It appears that the content of oxytocic principle approximates the amount of vasodepressor activity present. This agrees with the findings of Coon (1939), who proposed the use of the fowl vasodepressor response as the basis of a valid assay for the oxytocic principle.

TABLE 2

SUMMARY OF BLOOD-PRESSURE RESPONSES OF *Rana catesbiana* TO THE ADMINISTRATION OF PITUITRIN, PITOCIN, AND PITRESSIN IN DOSES OF 10 UNITS/KG

Injection	No. of Trials	Pressor Response		Depressor Response		No Response (No.)
		No.	Average (Mm Hg)	No.	Average (Mm Hg)	
Pituitrin.....	8	0	.....	8	−3	0
Pitocin.....	8	0	.....	8	−3	0
Pitressin.....	8	4	+3	1	−1	3

II. TOADS

The oxytocic fraction causes both antidiuretic and water-balance effects in frogs, but there are a few reports that indicate that this may not be true in toads. Novelli (1932) reported that pitressin was more effective than pitocin in causing water uptake by *B. arenarum*. Howes (1940), however, felt that pitocin was more effective in *B. bufo*. Recently, Jørgensen (1950) has demonstrated that the oxytocic fraction has much less effect on water balance and urine output in *B. bufo* than does the pressor fraction. Ewer (1951) has found a similar situation existing in *B. regularis*.

The Bermuda toad, *B. marinus*, shows a definite antidiuretic response to 0.01 unit/kg pituitrin. When pitocin and pitressin were administered to six toads at this dose, it became evident that the antidiuretic effect is present predominantly in the vasopressor fraction (Fig. 4). In this respect the toad is surprising in its resemblance to reptiles and mammals rather than to frogs, both in degree of sensitivity and in the antidiuretic re-

sponse to the vasopressor fraction.

Pasqualini (1938) found that inulin excretion was not diminished by the administration of pituitrin to *B. arenarum*. He concluded that the extract produced an increased reabsorption of water in the kidney of this species. Since such a tubular antidiuretic action has been demonstrated only in mammals and birds (Burgess *et al.*, 1933), it would seem unlikely that it exists in toads. Inulin and creatinine clearances on toads following administration of water and pituitrin injection failed to demonstrate the existence of a tubular antidiuretic effect. Pituitrin, 0.01 unit/kg, injected subcutaneously, causes a marked parallel fall in both inulin clearance and water output (Fig. 5). Seventeen clearance periods obtained on four toads both before and after pituitrin show that, at rates of urine formation varying from 0.068 to 0.979 ml/kg/min, there was a high degree of correlation between urine flow and glomerular filtration rate ($r = 0.933$). It appears that pituitrin produces a glomerular antidiuretic effect in the toad similar to that found in reptiles (Burgess *et al.*, 1933) and in the frog (Sawyer, 1951*b*).

The positive correlation between rate of urine formation and glomerular filtration rate in *B. marinus* is in agreement with the observation of Forster (1938) on *R. catesbiana*. He determined filtration rate over a comparable range of urine volumes. It appears quite certain that these amphibian species have a "reptilian-type" kidney, in that urine volume depends almost directly upon glomerular filtration. Forster found that creatinine and inulin clearances are identical in the bullfrog and concluded that neither was excreted by the tubules. In *B. marinus* the average ratio of creatinine to inulin clearance is 1.04 ± 0.066, which shows remarkable agreement, considering the fact that fecal contamination of urine specimens from the toads rendered some of the colorimetric determinations rather inaccurate. The concentration of creatinine or inulin in the urine was about twice that in the plasma. This is also in close agreement with Forster's findings on the bullfrog.

The toad showed such a marked antidiuretic response to low doses of mam-

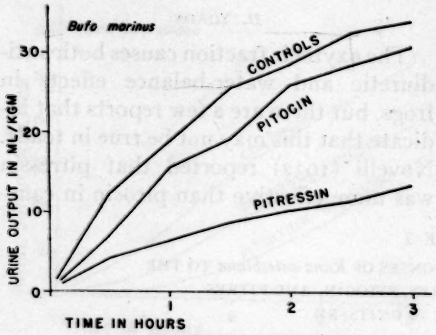


FIG. 4.—The effects of pitocin and pitressin, 0.01 unit/kg, on water excretion by the toad.

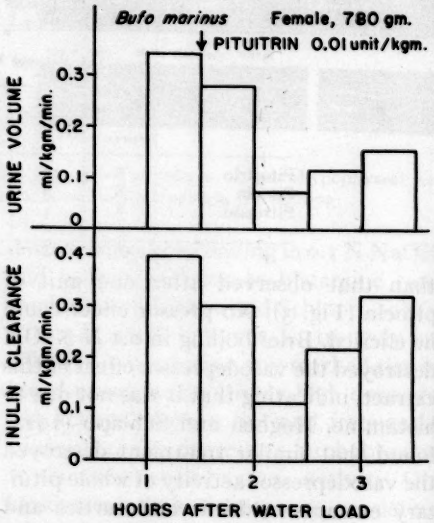


FIG. 5.—The effect of pituitrin, 0.01 unit/kg, on urine volume and inulin clearance in the toad.

malian vasopressor fraction that it was felt that it might show a sharp rise in blood pressure in response to pitressin. Doses as high as 20 units, however, failed to cause a pressor effect (Fig. 6). Pituitrin and pitocin, however, in a dose

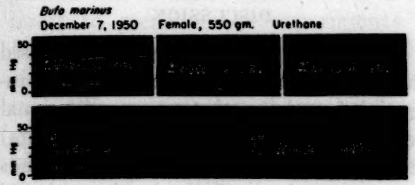


FIG. 6.—The effects of neurohypophyseal extracts on blood pressure in the toad.

principle for the alligator was examined. It appears that pitressin has much more antidiuretic effect than pitocin at the relatively low dose of 0.01 unit/kg (Fig. 7). Pituitrin is as effective as pitressin.

Simultaneous inulin and creatinine clearances were performed on four alligators before and after the administration of 0.01 unit/kg pituitrin. Urine collection was difficult, owing to the extremely small urine volumes following pituitrin, but twelve good clearance periods were observed. Inulin clearance always appears to be reduced in proportion to the reduction in urine flow. At rates of urine

TABLE 3

SUMMARY OF BLOOD-PRESSURE RESPONSES OF *Bufo marinus* TO THE ADMINISTRATION OF PITUITRIN, PITOCIN, AND PITRESSIN IN DOSES OF 5 UNITS/KG

Injection	No. of Trials	Pressor Response		Depressor Response		No Response (No.)
		No.	Average (Mm Hg)	No.	Average (Mm Hg)	
Pituitrin.....	7	0	.....	7	−7	0
Pitocin.....	7	0	.....	7	−6	0
Pitressin.....	7	0	.....	5	−3	2

of 5 units/kg regularly produced a depressor response (Table 3). The fall following pituitrin was approximately the same as that after pitocin, indicating that the pressor fraction present in the whole extract did not antagonize the depressor effect of the oxytocic fraction.

III. ALLIGATORS

Burgess, Harvey, and Marshall (1933) observed that pituitrin or pitressin cause an antidiuretic effect in the alligator. They found that glomerular filtration, as measured by xylose clearance, and urine output are depressed proportionally. Since such a glomerular antidiuretic effect in frogs is a property of the oxytocic fraction, the identity of the antidiuretic

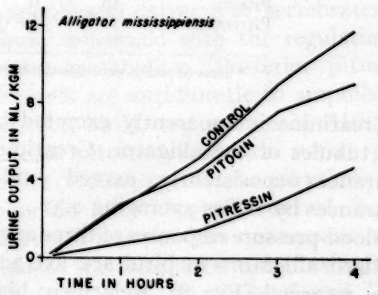


FIG. 7.—The effects of pitocin and pitressin, 0.01 unit/kg, on water excretion by the alligator.

formation of from 0.0023 to 0.031 ml/kg/min, there is good correlation between inulin clearance and urine volume ($r = 0.618$). This confirms Burgess, Harvey, and Marshall (1933), in that pitui-

trin causes a true glomerular antidiuretic effect in the alligator. The average concentration (inulin urine/plasma ratio) was 2.50. Burgess *et al.* found the average xylose urine/plasma ratio to be about 3.9 in their alligator.

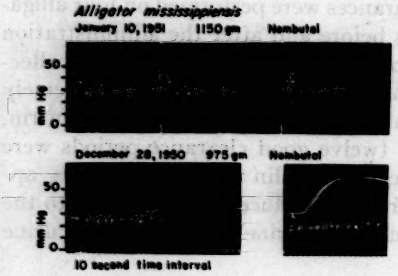


FIG. 8.—The effects of neurohypophyseal extracts on blood pressure in the alligator.

Creatinine is apparently excreted by the tubules of the alligator. Creatinine clearances consistently exceed inulin clearances by ratios averaging 2.31.

Blood-pressure responses of three anesthetized alligators to pituitary extracts were recorded (Fig. 8). Relatively high doses had to be employed to elicit definite responses. The depressor effect of pitocin and pituitrin is consistent. Pitressin causes a rise, a fall, or a diphasic response (Table 4), even in relatively huge doses. The depressor response is consistent with that observed by Hogben and Schlapp (1924) in the turtle after large doses of whole posterior lobe extract. Feakes *et al.* (1950) state that pitocin causes a fall and pitressin a rise in the blood pressure of a lizard, but they do not give the details of dosage used.

DISCUSSION

Krogh and Rehberg (1922) proposed that neurohypophyseal hormones are essential for the maintenance of normal vascular tone in the frog. Orias (1934) reported that hypophysectomized toads are hypotensive and that implantation of neurohypophyses or injection of extracts raises the blood pressure. Hogben and Schlapp (1924), however, found that relatively large doses of whole posterior pituitary extract cause, at best, a small, slow rise in blood pressure of frogs. They also observed a slow fall in turtle blood pressure following injection of the same extracts. They concluded: "Pituitary extracts do not produce perceptible effects on the blood-pressure of the reptile and amphibian, when administered in quantities which are of an order of magnitude to which physiological significance could be justifiably attached."

The blood-pressure experiments on frogs, toads, and alligators reported here bear out the conclusions of Hogben and

TABLE 4

SUMMARY OF BLOOD-PRESSURE RESPONSES OF THE ALLIGATOR TO THE ADMINISTRATION OF PITUITRIN, PITOCIN, AND PITRESSIN IN DOSES OF 5 UNITS/KG*

Injection	No. of Trials	Pressor Response		Depressor Response		No Response (No.)
		No.	Average (Mm Hg)	No.	Average (Mm Hg)	
Pituitrin.....	8	1	+1	8	−2	0
Pitocin.....	10	0	.....	10	−4	0
Pitressin.....	11	2	+2	8	−1	2

* Some responses were diphasic and appear in more than one column.

Schlapp. Relatively large doses of vasopressor fraction are necessary to produce a pressor response in the alligator or bullfrog. No pressor response could be elicited in the toad. All three animals show consistent vasodepressor responses to pitocin and to whole extract. It is of interest to mention the experiments of Feakes *et al.* (1950) on the duck-billed platypus, *Ornithorhynchus*. They found that this monotreme shows responses qualitatively similar to those seen in the bird (Coon, 1939) and in the alligator. Pitocin evokes a fall, pitressin a rise, and the whole extract a diphasic response. The doses necessary to produce these responses were slightly larger than those necessary in the chicken but considerably smaller than those needed in the alligator. It becomes evident that the pattern of response is the same in amphibians, reptiles, monotremes, and birds, but the sensitivity increases as one passes from cold- to warm-blooded vertebrates.

The low sensitivity to the peripheral vascular effects of mammalian extracts shown by reptiles and amphibians may merely reflect the fact that these are hormones of protein-like nature and may be very different from the natural hormones in these classes. Heller (1941*b*) has found that the frog pituitary is relatively rich in water-balance activity (assayed on frogs) while poor in antidiuretic and oxytocic activity (assayed on mammals). Jørgensen (1950) has shown that 1/200 of a frog or toad pituitary causes a definite water-balance effect in *B. bufo* approximately equivalent to the effect of 0.01 unit of mammalian extract.

That the low peripheral vascular sensitivity of reptiles and amphibians to mammalian neurohypophyseal extracts may result merely from the use of foreign hormone is not supported by the experiments reported on alligators and toads. Both these species show extreme sensitivity to the glomerular antidiuretic effect of pitressin. Both respond to subcutaneous doses of 0.01 unit/kg with prolonged oliguria. Doses as high as 20 units/kg injected intravenously, however, fail to produce consistent pressor effects. It would seem unlikely that species specificity would be so important in one vascular end-organ—the peripheral blood vessel—and not in another—the afferent glomerular arteriole.

In *R. catesbiana* ½ pituitary from a bullfrog injected intravenously causes a very slight fall in blood pressure, considerably less than that caused by 1 unit of pitocin. The fall produced is consistent with the oxytocic activity of the extract, which was found to be 0.35 unit per pituitary when assayed on the rat uterus. The blood-pressure effect of the frog extract on the frog appears to differ in no way from that produced by mammalian extracts. If endogenous neurohypophyseal secretion plays an important role in maintaining blood pressure, it seems surprising that ½ bullfrog pituitary has such a negligible effect on the circulation of the bullfrog.

The most consistent effects of neurohypophyseal extracts in vertebrates are those concerned with the regulation of water metabolism. Posterior pituitary extracts are antidiuretic to amphibians, reptiles, birds, and mammals. Although teleost pituitaries contain active principles (Heller, 1941*a*), the part these may play in physiological control is not known. Pituitrin does not appear to influence water balance in fresh-water fish (Burgess *et al.*, 1933; Boyd and Dingwall, 1939). Euryhaline teleosts, such as the eel, have not, however, been investigated. Such forms must have highly developed means for varying urine flow to compensate for the osmotic strains on homeostasis inherent in their mode of life. The glomerular antidiuretic effect

would seem ideally suited for such regulation and may well be involved.

The glomerular antidiuretic effect is present in amphibians, reptiles, and birds. The toad and alligator resemble mammals, in that they show a profound antidiuretic response to the administration of relatively small doses. The frog, however, is not so sensitive (Table 5). The toad and most reptiles probably depend upon glomerular antidiuresis as a physiological mechanism for the conservation of body water. Frogs usually stay within reach of fresh water and appear to rely more upon the effect of neurohypophyseal principles on water uptake. Pituitary extract produces an increase in the permeability of the skin to water—the water-balance response—at doses that are not antidiuretic (Sawyer, 1951*a*). The glomerular antidiuretic response may well be of little importance in the normal maintenance of fluid balance in the frog.

Some of the known effects of neurohypophyseal extracts in vertebrates are summarized in Table 6. The fraction responsible for each response is indicated. It appears that the antidiuretic effects are chiefly properties of the vasopressor fraction; the vasodepressor effect is a property of the oxytocic fraction. The chloruretic effect appears to be due to the oxytocic fraction in the frog and the rat.

TABLE 5

SUMMARY OF THE EFFECTS OF INJECTION OF PITOCIN AND PITRESSIN ON URINE OUTPUT IN BULLFROG, TOAD, AND ALLIGATOR

Animal	No. of Animals	Dose of Extract (Units/Kg)	Urine Output (Ml/Kg/3 hours)		
			Controls	Pitocin	Pitressin
R catesbiana	6	10	44.5±2.30	17.4±2.46	35.8±6.12
B marinus	6	0.01	33.8±4.63	30.7±5.20	13.4±3.27
Alligator	6	0.01	9.6±1.58	7.6±1.47	2.8±0.92

TABLE 6

DISTRIBUTION OF RESPONSES TO POSTERIOR PITUITARY FRACTIONS IN VERTEBRATES*

Animal	End-Organ					
	Skin	Blood Vessels			Renal Tubule	
	"Water Balance"	Glomerular Antidiuretic	Vasopressor	Vasodepressor	Tubular Antidiuretic	Chloruretic
Frog	Pitocin	Pitocin	Pitressin	Pitocin	None	Pitocin
Toad	Pitressin	Pitressin	None	Pitocin	None	?
Alligator	*None*	Pitressin	Pitressin	Pitocin	None	?
Chicken	*None*	*Pitressin*	*Pitressin*	*Pitocin*	*Pitressin*	?
Rat	*None*	?	*Pitressin*	*None*	Pitressin	Pitocin

* Italicized entries in the columns represent opinions from the literature (see text for references); the remainder of the entries are based, at least in part, on personal experience.

These differing effects of the specific fractions may be of pharmacological interest only, since it appears likely that the neurohypophysis is capable of secreting only one large hormone containing all activities. If this is true, it would seem awkward if the secretion exerted several actions on different end-organs, producing effects which lacked a common purpose. It might be unfortunate if a need for water conservation was met not only by antidiuresis but also by a sharp rise or fall in blood pressure and a strong contraction of the uterus and the intestinal muscles.

The conservation of body water is the basic need that could be satisfied by neurohypophyseal secretion in amphibian and terrestrial vertebrates. Chloruresis is not inconsistent with antidiuresis, since an increase in plasma osmotic pressure appears to be the stimulus for posterior pituitary secretion (Verney, 1947). Increased excretion of salts, as well as retention of water, would help restore homeostatic equilibrium under such circumstances.

The glomerular antidiuretic effect appears to be the most widely distributed response to neurohypophyseal extracts and must be considered the most primitive yet described. It is essentially an effect on smooth muscle, the smooth muscle of the afferent glomerular arteriole. Thus, from an evolutionary standpoint, posterior pituitary extracts in high enough doses might be expected to influence vascular smooth muscle in general. This may explain, in part, the striking vasopressor effects seen in anesthetized mammals, effects that appear purely pharmacological in importance. Whether the oxytocic response represents a secondary adaptation of another smooth muscle to respond to these principles cannot be determined until the physiological role that these hormones may play in parturition is further elucidated.

During vertebrate evolution a series of adaptations to neurohypophyseal secretion has taken place. These may be correlated with habitat (Fig. 9). Howes (1940) has shown that the tadpole is unable to respond to the water-balance effect of pituitrin but gains competence during the period of tail resorption. The frog, usually living in the proximity of fresh water, has a well-developed water-balance response, allowing an increased osmotic inflow of water from the hypotonic external medium across the skin. The frog is, however, relatively insensitive to neurohypophyseal effects on urine excretion. The toad, however, is extremely sensitive to glomerular antidiuretic, as well as to water-balance effects, and has apparently gained a further degree of emancipation from dependence upon the pond. Two thoroughly successful terrestrial classes—reptiles and birds—have combined uricotelic nitrogen metabolism and the glomerular antidiuretic effect into an extremely efficient means for the conservation of water even under the most arid conditions.

The tubular antidiuretic effect represents the adaptation of another end-organ, of entirely different anatomical nature, to serve the same general purpose. The renal tubule of mammals and, to a lesser degree, of birds has gained competence to respond to neurohypophyseal secretion in a unique manner, by the active transport of water. Such a mechanism became necessary if mammals, handicapped as they are by the absence of uricotelism and the presence of a high metabolic rate, are to live successfully under terrestrial conditions. It is interesting to note that marine seals (Hiatt and Hiatt, 1942) and the desert kangaroo rat (Schmidt–Nielsen, 1950) appear

to supplement the tubular antidiuretic effect by decreasing glomerular filtration rate as a secondary defense of fluid balance. The pituitary gland of the kangaroo rat contains and secretes much more antidiuretic activity than does that of the white rat (Ames and Van Dyke, 1950).

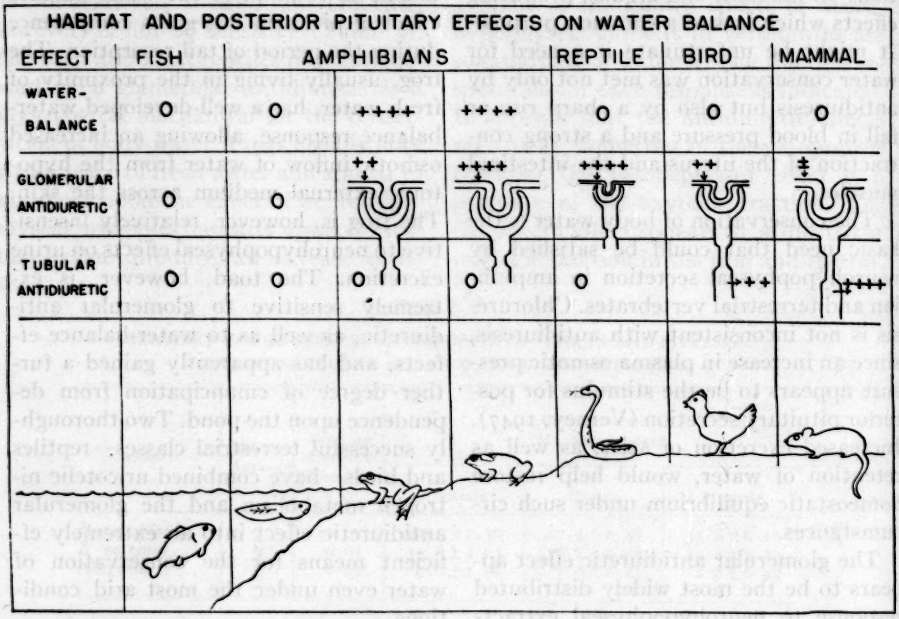


FIG. 9.—Evolutionary distribution of responses to neurohypophyseal principles related to water metabolism.

It is very possible that in such forms the posterior pituitary principles regulate glomerular filtration rate as well as the extraordinarily well-developed tubular reabsorption of water. Thus the glomerular antidiuretic effect may not have been entirely lost in the evolution of mammals from reptiles.

The evolution of posterior pituitary effects on water metabolism comprises an excellent example of the adaptation of several differing tissues to respond to a single hormone. The tissues may be very different and may each respond in a different manner. The hormone, however, appears to remain essentially the same. The stimulus for its secretion—namely, dehydration—and the result of its secretion—the restoration of body water—also appear unchanged in amphibian and terrestrial vertebrates.

SUMMARY

1. The distribution of responses to the fractions of posterior pituitary extracts among representative vertebrates has been investigated.

2. In frogs the oxytocic fraction exerts glomerular antidiuretic, "water-balance," chloruretic, and vasodepressor effects. The vasopressor fraction can produce a weak pressor response. An extract of bullfrog pituitaries shows a very slight vasodepressor effect on the bullfrog, consistent with the small amount of oxytocic activity present.

3. In toads very small doses of the vasopressor fraction produce both glomerular antidiuretic and water-balance effects. The oxytocic fraction exerts a consistent vasodepressor effect. No pressor response could be demonstrated.

4. In the alligator the vasopressor fraction exerts a marked glomerular antidiuretic effect but a very slight vasopressor effect. The oxytocic fraction elicits a vasodepressor response.

5. On the basis of the phyletic distribution of responses, it is concluded that the effects related to the regulation of body water are important homeostatic mechanisms in amphibians, reptiles, birds, and mammals. These effects are the glomerular antidiuretic, tubular antidiuretic, and water-balance effects. The effects on blood pressure are so slight in lower vertebrates that it appears highly improbable that they are of importance in the maintenance of normal vascular tone.

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GROWTH RATE OF THE WHITE RAT IN RELATION TO NUMBER PER CAGE[1]

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WITH THE TECHNICAL ASSISTANCE OF MISS FRIDA BERGMANN

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GROWTH rate is commonly used as an index of the response of an animal to any treatment, even if it has no direct nutritional implications. Most of the experiments are carried out with animals housed in different cages, and sometimes some of them are put into individual ones to be studied thoroughly, while the others remain in larger groups receiving the same treatment. Usually it is not realized that different numbers per cage affect growth rate to some extent and may become a bias unless due attention is paid to it.

Vetulani (1931) noted in mice that males grew better in groups of 3–4, followed by those in groups of 5–6, 9–12, and 1. No significant difference could be observed in females. Retzlaff (1939) obtained the same results when the animals lived at low temperatures, 15° C.; but animals at 20° C. grew better when alone. It was concluded from this that the actual cause of the difference was the maintenance of body temperature, which was easier for these small animals if they were assembled in medium-sized groups. But when the animals were helped to keep their own body temperature by raising the environmental one to 20° C., another set of causes entered into action, and the single animals grew better.

As the influence of temperature had already been established by Retzlaff, the present experiments were planned in order to establish the causes acting in favor of the single animals when environmental temperature is kept above 22° C.

PROCEDURE

Experiment No. 1.—Fifty white rats, 25 males and 25 females, with weights ranging from 78.3 to 98 gm. in males and 75–97 gm. in females, were divided into

[1] I want to express my gratitude to Professor W. C. Allee, who made available previous information on this subject, and to Dr. J. T. Lewis for his kind encouragement and help.

the following groups: two of 1, two of 2, one of 6, and one of 13 of each sex per cage. They were put into common cages of the rat colony, which measured 39 × 39 × 34.5 cm. All the cages referred to in this paper have wire floors; therefore, the animals, even the most crowded, were clean. Scarcity of animals

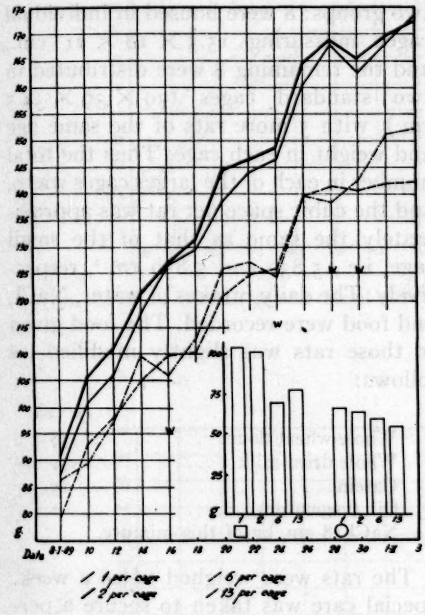

FIG. 1.—Mean growth rate of rats at densities of 1, 2, 6, and 13 per cage. *W* indicates lack of water. The columns show the absolute mean increase of body weight for graphs.

prevented better planning. This was actually a preliminary experiment, and no individual records of the animals were kept. The experiment lasted 26 days, and the animals were weighed every other day. Room temperature varied from 21° to 31° C. Figure 1 shows the improvement of the different groups, composed of both sexes. Water was supplied in tins which were easily upset by the rats, and it can be seen in the figure that, when water was missing, groups of larger numbers suffered more than the others.

There is a negative correlation coefficient between numbers per cage and growth rate of −0.764 for males and −0.48 for females. On account of the small number of degrees of freedom (4), neither these correlations per se nor the difference between them are significant. As they were not weighed individually, no other statistical analysis was possible. Although the animals were distributed at random, groups of lower numbers were favored by initial higher weights. This may bias the results; nevertheless, they are not very different from those of Experiments 3, 4, and 5, where the opposite situation was secured at the start.

It can be concluded that, while no important difference exists between 1 and 2 per cage and 6 and 13, less crowded groups grow better than larger ones.

Experiment No. 2.—Animals from 28 up to 35 days old, with a mean weight of 29.3 gm. for males and 28.9 gm. for females, were used. They were divided into two groups of 1, two of 2, one of 4, one of 6, and one of 12 of each sex. Water was supplied in self-feeding bottles, and the animals were given the standard food of the rat colony, as follows:

	Per Cent
Whole-wheat flour	68
White wheat flour	14
Casein	5
Whole dried milk	10.4
Oil (vegetable)	1.7
NaCl	0.9

Cod-liver oil was given once a week and green vegetables daily. Special care was taken to supply an abundant amount of both water and food to all the groups. The experiment lasted 8 months. Five animals died at the beginning and were replaced by others with the nearest possible weight. One of the group of 12 males died toward the end and was not

replaced. The temperature ranged from 22° to 24° C.; in a few instances it rose to 28° or fell to 19° C. Figures 2*A* and 2*B* show the weight improvement of these groups. Again the columns show the absolute gain in mean body weight.

Statistical analysis (analysis of variance) showed a significant difference between the weight increase of groups ($F = 4.654$, $p < 0.01$ for males and $F = 2.87$, $p < 0.05$ for females), but no significant interclass correlations between number per cage and increase of weight were found (for males $r = -0.208$ and for females $r = -0.35$). There is no significant difference among groups of 4, 6, and 12 either in males or in females ($F = 1.60$ for males and 1.33 for females, the 0.05 significance level being 3.55 and 3.52, respectively). Since a definite difference among groups is found, this lack of a significant negative correlation can be attributed either to chance (the group of 12 had a larger increase than the groups of 4 and 6 in males) or to an actual lack of correlation, since the effect of crowding may be complete when there are 4 animals per cage. This last hypothesis seems more probable.

Experiment No. 3.—This experiment was planned in order to reduce male variability. Twenty males were divided into two groups: 10 were distributed in ten common cages and 10 in one. Each rat of the 1's had a litter-brother in the group of 10. The mean weight of the first group was 54.5 gm., and that of the second was 57 gm. The last one was favored, for it was expected that the single ones would grow better. As can be seen in Figure 3, the 1's had exceeded the group of 10, 37 days after the beginning of the experiment. At the end the difference is significant: $t = 3.73$, $p < 0.01$. The experiment lasted 6 months. At this moment only 2 of the group of 10 weighed 1 and 2 gm. more than their respective brothers in the 1's group, the mean difference being 28 gm.

Experiment No. 4.—This and the next experiment were carried out in order to ascertain the cause of this differential growth. Four groups of 4 males each, 35–36 days old, were taken from the same number of litters and divided into two groups: 8 were housed in individual cages measuring 15.5 × 18 × 21 cm., and the remaining 8 were distributed in two standard cages (39 × 39 × 34.5 cm.), with 5 more rats of the same age and weight in each cage. Thus the total number in each of the larger cages was 9, and the cubic space per rat was approximately the same as that of the small cage, i.e., 5,830 and 5,859 cm.^{3}, respectively. The daily intakes of water, NaCl, and food were recorded. The food given to those rats was slightly modified, as follows:

	Per Cent
Whole-wheat flour	78
Whole dried milk	10
Casein	10
Oil (vegetable)	2

NaCl 18 gm/kg of this mixture.

The rats were weighed twice a week. Special care was taken to secure a permanent supply of water and food. Figure 4 (lines bracketed as *1*) shows the growth record. This experiment lasted only 1 month; the difference between single rats and groups was, however, highly significant: $F = 11.48$ ($F_{0.01} = 5.95$). In the statistical analysis no account was taken of the 5 rats added to each group to increase its number up to 9. As can be seen in Table 1, single rats eat more than those in groups and need less food to produce the same weight gain. No important difference was found in the intake of water and NaCl.

Experiment No. 5.—This experiment was designed to see whether the renewal

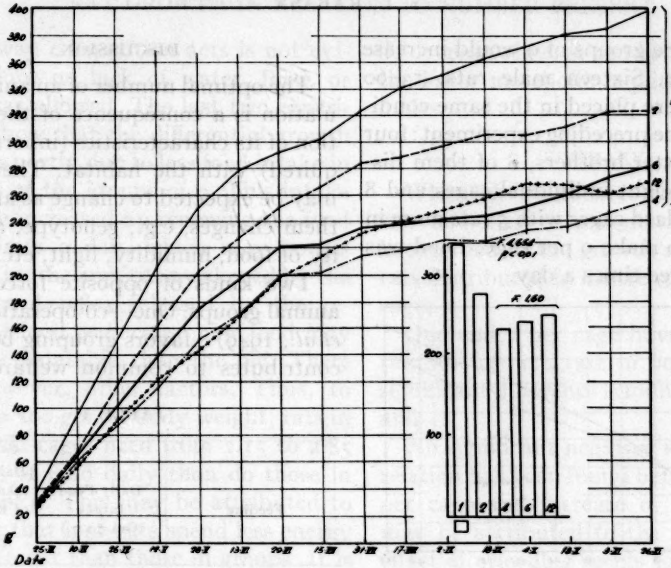

FIG. 2*A*.—Mean growth rate of male rats at densities of 1, 2, 4, 6, and 12 per cage

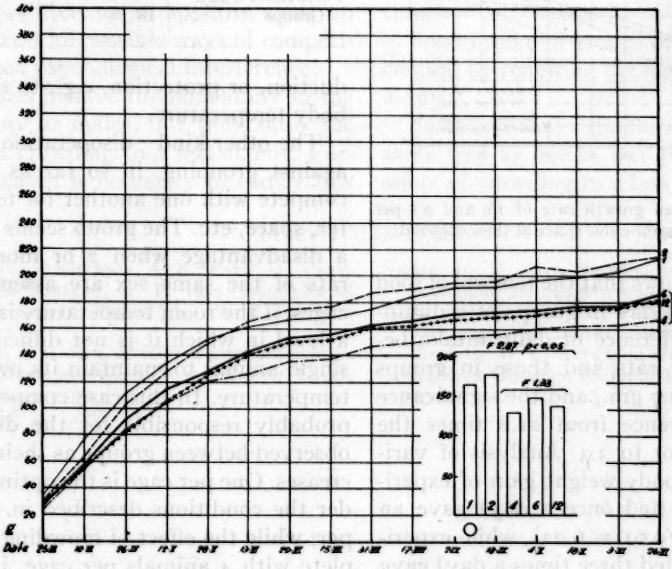

FIG. 2*B*.—Mean growth rate of female rats at densities of 1, 2, 6, and 12 per cage

of food in the groups of 9 would increase their intake. Sixteen male rats, 53–60 days old, were placed in the same conditions as in the preceding experiment: four groups of litter-brothers, 8 of them distributed in eight individual cages and 8 in two standard cages with 5 rats more in each one, to make 9 per cage. Food was changed three times a day.

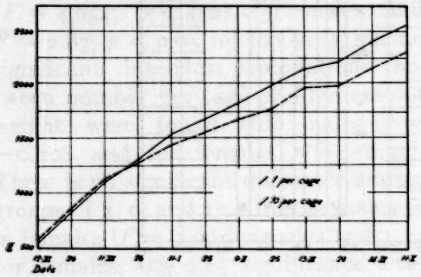

FIG. 3.—Mean growth rate of litter-brothers housed at a density of 1 versus 10 per cage.

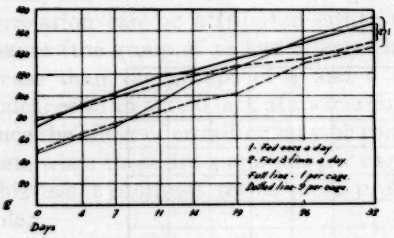

FIG. 4.—Mean growth rate of 1's and 9's per cage having the same cubic space at their disposal.

Table 1 shows that the renewal of food three times a day in groups of 9 diminished the difference of daily intake between single rats and those in groups from 6.1 to 2.7 gm., and the significance of the difference from 21.8 times the standard error to 3.3. Analysis of variance of the body-weight gain of experiment No. 4 (fed once a day) gave an $F = 11.48$ ($F_{0.01} = 6.22$), while experiment No. 5 (fed three times a day) gave an $F = 5.8$ ($F_{0.01} = 5.95$) (see Fig. 4, the lines bracketed as *2*).

DISCUSSION

The optimal number of an animal population is a consequence of the interaction of its characteristics (inborn and acquired) with the habitat. Therefore, it may be expected to change as any one of them changes, e.g., genotype, availability of food, humidity, light, etc.

Two kinds of opposite forces act in animal groups. One—co-operation (Allee *et al.*, 1949)—favors grouping because it contributes to common welfare, reproduction, or protection, e.g., to maintain body temperature.

TABLE 1

Feeding	Daily Food Intake (Gm.±s.e.)	Daily Food Intake per 100 Gm. of Body-Weight Gain (Gm.±s.e.)
Once a day:		
Single rat	21.55±0.279	16.93±0.35
Groups	15.45	19.08
Once a day:		
Single rat	21.45±0.812	20.65±0.817
Three times a day:		
Groups	18.75	23.5

The other kind—disoperation—works against grouping, in so far as animals compete with one another for food, water, space, etc. The group seems to be at a disadvantage when 2 or more white rats of the same sex are assembled in cages, if the room temperature is kept at a level in which it is not difficult for a single animal to maintain its own body temperature. In this case competition is probably responsible for the difference observed between groups as their size increases. One per cage is the optimum under the conditions described in this paper, while the effect of crowding is complete with 4 animals per cage, irrespective of the sex. Larger groups fail to show a significantly greater effect.

The way competition acts is not evident, since no lack of water, food, or space was allowed. The last two experiments show that the differential growth rate was partly due to less food intake in the rats of the groups of 9. This intake could be increased by renewing the food three times a day, which may be explained by the appetite awakened by the conditioned reflex released by the renewal of food or because new food may be more palatable than the old. There are, however, other factors. Thus, to build up 100 gm. of body weight, rats in individual cages need from 2.15 to 2.85 gm. less of food daily than do those in groups of 9. This may be attributed to the fact that lone rats spend less energy in movement than those in groups. It is not easy to ascertain why it is not possible to obtain the same food intake, irrespective of the number per cage. Perhaps if food was changed more frequently, they would eat more, but probably the difference in appetite sums up and reflects all possible ways of competition, even psychological interference.

Females behave fundamentally in the same way as males, but they show the effect of crowding to a less extent. Further research is necessary to clarify this field; meanwhile, it can be attributed to the lower metabolic requirements of females.

SUMMARY

When environmental temperature is kept above 22° C., a significant difference is observed in the growth rate of young rats distributed in different numbers per cage.

One and 2 per cage have always had the best growth rate in both sexes. No significant difference is found among 4, 6, and 12.

No significant negative interclass correlation has been found between number per cage and increase of weight. This may be attributed to the fact that the effect of crowding seems to be complete with 4 animals per cage and does not show any greater effect in larger groups.

Rats in groups of 9 eat less than those kept alone and need more food to build up 100 gm. of body weight in the same time.

Food intake of groups of 9 may be increased by renewing the food three times a day.

Females behave fundamentally in the same way as males, but they show the effect of crowding to a less extent.

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NEW BOOKS IN PHYSIOLOGICAL ZOÖLOGY

[This section comprises reviews of selected new books containing new and significant material in the field of physiological zoölogy. Books submitted for review should be sent directly to the review editor: Frank A. Brown, Jr., Department of Zoölogy, Northwestern University, Evanston, Illinois.]

COMPARATIVE ANIMAL PHYSIOLOGY,[1] BY C. LADD PROSSER AND OTHERS

Reviewed by EDWARD F. ADOLPH
University of Rochester, Rochester, New York

COMPARATIVE physiology is a science without form but with the prospect of having one. Its virtue as well as its curse is that its possibilities are manifold. No two physiologists have the same view of its future. Comprising a grand panorma which admirers of animals have surveyed, this science is at least as old as Aristotle (*De partibus animalium*). A host of new materials for it was uncovered by Reaumur, Trembley, and others in the eighteenth century. It began to acquire system in the hands of Johannes Müller and his successors in the nineteenth century, when it came to consist largely of inferences from comparative anatomy. Only in the twentieth century did it stand on its own substance of experiment and quantitative study, being assembled comprehensively by Winterstein (1910), Buddenbrock (1937), and more briefly by Hogben (1926), Jordan (1929), Rogers (1938), Yapp (1939), and Scheer (1948).

Today the science of comparative physiology is at last represented in a volume that can be thought to decide for some time the form approximating which the science will mold itself. This volume, written by a group of five American physiologists (David W. Bishop, Frank A. Brown, Jr., Theodore L. Jahn, C. Ladd Prosser, and Verner J. Wulff), is a comprehensive treatise of the subject. "Comparative animal physiology," they say, "is concerned with the ways in which diverse organisms perform similar functions. . . . While other branches of physiology use such variables as light, temperature, oxygen tension, and hormone balance, comparative physiology uses, in addition, species or animal type as a variable for each function." No longer need comparative physiology be regarded as the refuse after human physiology has appropriated whatever it could assimilate.

In each corner of physiology, comparative physiology arrived when the functional differences among species found significance. Significances first appeared in phenomena of behavior and responses. They consisted in the realization that no other set of responses could substitute for those that existed. Later the differences were traced in organs, tissues, and cells, in that order. Gradually the terms "curious," "primitive," "peculiar," and "aberrant" disappeared from the biologist's vocabulary, as it was realized that no animals were merely imperfect mammals. Tolerance of variety in the world of animals led to beginning appreciation of the unique combinations of functions whereby each type of organism lives successfully.

Today the comparative physiologist can be proud of certain achievements, such as his comprehension of the variety of manifestations that are based on common physical and biochemical ground plans. But he can also be humble at his inadequacies in describing the variety which is his choice for study. Future progress must depend upon multitudinous inventions of schemes for recognizing and correlating unit differences that emerge from underlying similarities. No longer can type samples of animals satisfy the need for seeing, at one time, the broad outlines and the minute details by which "particularities" appear as the special endowments of organisms.

Study of any chapter in the present work impresses the reader with the remarkable variety of manners in which life-processes occur in animals. Thus organisms from *Arbacia* eggs to whales extract water from the environment and retain it in their bodies in classifiable ways. Yet the variety of ways is not infinite; it has both limitations and physical reasonableness. Functions that seemed "a priori" impossible to the earlier scientists are known to have been achieved by animals; and now it is plain that no

[1] Philadelphia: W. B. Saunders Co., 1950 Pp. x+888. $12.50.

principles established in other sciences have been flouted in the processes employed.

Each chapter of the book considers a group of functions as ordinarily defined, such as "feeding and digestion," "temperature relations," "chemoreception," and "nervous systems." More than half of the 23 chapters were written by the leading author; by the others a uniformity of treatment has been achieved. The means by which each group of functions in question is carried on are classified, the best-understood examples of each class are described, and comparisons among classes are drawn. General familiarity by the reader with 200 or 300 genera of animals in all phyla is assumed.

Over 300 figures, portraying both structural relations and graphical materials, support the compressed text. Most figures are redrawn in a clear manner. Over 70 tables gather and coordinate information from a host of sources. They in themselves constitute keys to an intricate subject. Each chapter has its own list of references, which are preponderantly to American journals, and all together number 3,500. From those references have been extracted concise statements of results, and those statements have been woven into a remarkably readable and logical set of chapters. Conclusions drawn are properly tentative, yet definite.

The authors hope that the book will serve both as a textbook and as a sourcebook. The latter hope is already fulfilled. As a textbook it remains to be tested. For advanced students it may prove to be a guide in a wilderness. For undergraduate students one can only hope that the invitation to explore which this book contains will not be masked by the very wealth of its materials.

A reviewer can easily find points in which he would have worked differently from the authors. I would have asked for more emphasis on experimental methods, for more appreciation of the historical approach to some topics, and for a final chapter that would point out more comprehensive comparisons than could be mentioned in the present chapters. Perhaps I can even wish for a précis by which inspirable undergraduates could obtain a vision of the grand themes and gorgeous interrelations in animal functions. But these requests visualize additions to what is already an overflowing book. I would have asked the publishers to furnish less transparent paper and to help ferret out the few remaining typographical errors.

Whatever comparative physiology accomplishes in this century, it will accomplish sooner and better because of this book. Workers in the field and many outside it are beholden to the book. Few physiologists can afford to remain outside its sphere of influence.

ECOLOGY OF ANIMAL PARASITES,[1] BY JEAN G. BAER

Reviewed by C. G. GOODCHILD
Southwest Missouri State College

SELDOM has any large field in the biological sciences been rewarded with so many timely books in so short a period of time as has parasitology. Clinical and veterinarian texts were formerly plentiful but were painfully lacking in breadth and scope for general analysis of the biological aspects of parasitism. Within the past few years, however, several noteworthy books have appeared from the hands of capable specialists.

Jean G. Baer, professor of zoölogy, University of Neuchâtel, has produced another of these modern books, presumably from lecture materials which he used in two courses in parasitology given as a visiting professor in the Graduate College, University of Illinois. Physically the book has a pleasing format, with clean-cut double columns on its 224 pages and 162 generally excellent line-cut illustrations.

In addition to an Introduction and an adequate Index, there are five major sections dealing with the following fundamental parasitological problems: animal adaptations, definitions; adaptations to parasitism; host-parasite relationships; physiology of parasites; origin of parasitism. The author is to be commended for adopting rigid ecological terminology and for using it consistently throughout his treatise. Precise words—"phoresis," "commensalism," "symbiosis," and "parasitism"—as defined and used by Baer, should be in the working vocabulary of every parasitologist.

The section dealing with adaptations to parasitism is the longest and best in the book. Several chapters on Mollusca, annelids, Arthropoda (crustaceans especially) and Pentastomida should be required reading for parasitologists whose present interests are limited to protozoans, helminths, or insects. However, unexplained and inexcusable slightings or omis-

[1] Urbana: University of Illinois Press, 1951. Pp. x+224+162 figs. $5.00.

sions occur for the Turbellaria and Aspidobothria. From the former have evolved "some of the most successful lines of parasites" (p. 212); accordingly, they require additional summarization and theorizing to afford for them the proper perspective.

Remaining sections have critical evaluations of their field but are not so crucial to the professional parasitologist because similar or identical topics are available in comprehensive original research or review articles in the periodical literature. Nevertheless, it is well to have such a summary compacted for ready reference and for student perusal. Furthermore, lists of references at the ends of chapters or sections, while not exhaustive, include most of the important recent papers, and papers or books in which noncited references may be found are italicized. Excellent also are figures of possible life-history pathways and phylogenetic trees, showing possible relationships within and between parasitic groups.

Minor criticisms of the book include: (1) Difficulties with italics: *Sacculinae* (p. 63), *Rhizocephala* (p. 63), but "rhizocephala" (p. 174); *lycophorae* (p. 137), but "lycophorae" (p. 138)—all used not in a generic but in a group sense; *Hymenoptera* (p. 83), but "diptera" (p. 78). (2) Inadequate proofreading: page 67, paragraph 3, should read *in vitro* for *in vivo* (?); Figure 135 is upside down; Figure 142 has a line from "adult" to "miracidium" which is unintelligible (are eggs omitted here?); page 188, "Moreover, their symbiotic fauna tends to indicate that such families as the camel and llama . . ."! (3) Errors in fact: page 79, "Where larval flies occur in practically every type of environment that is capable of supporting life, except sea water"—culicids, chironomids, and others swarm from the ocean in disproof of this statement; page 111, "All flatworms have ectolecithal eggs . . ."—at least two major groups in the Turbellaria do not have ectolecithal eggs (cf. Hyman, *The Invertebrates*, 2: 119); page 125, invariably first intermediate hosts include only snails, lamellibranchs or scaphopods—this unequivocal statement depreciates *Hydroides dianthus*, a polychaete annelid, as an exceptional host; page 166, "The complete absence of characteristic genera of digenetic trematodes from elasmobranchs . . ."—this statement is an error of fact and constitutes a disconcerting omission of one large digenetic trematode subfamily, the Anaporrhutinae, found almost exclusively in elasmobranchs and certainly with characteristic genera in them. (4) A repetitious and mentally blocking use of the word "so-called" which forces the reader to analyze its use in each instance.

The book is crammed with provocative parasitological concepts and speculations. It is fruitful for ideas for further research into unknown but desperately needed facts of parasitism. Physiologists will glean many ideas worthy of investigation. Ecologists will be amazed, perhaps shocked, at the morphological, physiological, and speculative biology presented in a textbook of "ecology." Geneticists will mull over such statements (p. 188) as "It seems difficult to imagine that anaerobic organisms arose by mutations since, even if this were possible, they would invariably die unless such mutations were caused by the environment, by their presence in the paunch of ruminants." They will also despair of the neo-Lamarckism implied in the fifth paragraph on page 212: "Parasitism affects an organism in two totally different ways, physiologically, through causing it to lose the power to synthesize part or all of its essential nutritional requirements, and morphologically, through the loss of all superfluous organs. The greater the effect of either one or both of these factors, the more highly specialized and therefore the more intimately adapted to its host will the parasite be." Biologists will read the last sentence in the book with great disquietude because, unqualified, it is not true: "As new hosts appeared and new niches became available, the parasites also invaded them, yet in the process they have continually been subjected to the same laws as all other living organisms, except that the rather peculiar nature of their ecological niches excludes competition and, consequently, parasites would be the only animals that are spared the struggle for existence."

THE INVERTEBRATES,[1] VOL. II: *PLATYHELMINTHES AND RHYNCHOCOELA* VOL. III: *ACANTHOCEPHALA, ASCHELMINTHES, AND ENTOPROCTA*, BY LIBBIE H. HYMAN

Reviewed by F. A. BROWN, JR.
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Now, after about a ten-year interval since the appearance of Volume I, *The Invertebrates: Protozoa through Ctenophora*, there have appeared Volumes II and III of the projected series. These volumes comprise the first extensive treatment of the Invertebrates to appear in the English language for nearly half a century.[2] They are arriving at a period in the history of zoölogy when the invertebrates are becoming more and more neglected in the training program of the professional biologist. Dr. Hyman has done a superb job in selecting from the literature a vast amount of material over all phases of the biology of the invertebrates and organizing it into very readable form and one arranged to serve most effectively as a reference source. Volumes II and III maintain the general organizational plan and high quality of the first one.

In Volume II the first fifty-one pages are devoted to a general consideration of the Bilateria, those phyla possessing fundamental bilateral symmetry. There is a critical evaluation of the three prevalent theories of origin of the Bilateria from radiate ancestry, namely, the ctenophore to polyclad, the ctenophore to trochophore, and the planuloid to acoeloid. The conclusion is reached that only the last is tenable at this time. Then such general phenomena as spiral cleavage, determinate development, and trochophoral types, so characteristic of the protostomate branch of the invertebrates, are briefly and lucidly described and are discussed from the standpoints of phylogenetic relationships. Next there are excellent brief accounts of the diverse methods of mesoderm and coelom formation among animals. The four theories of the phylogenetic origin of the coelom, namely, the enterocoel, gonocoel, nephrocoel, and schizocoel theories, are discussed and evaluated. This is followed by an interesting discussion of the origin of segmentation. This section of the volume concludes with a general description of organ systems of the Bilateria, in which effort is directed chiefly at attempting to reduce to as few terms as possible the synonymity which has gradually grown up among the different invertebrate phyla for entirely comparable structures. Interesting suggestions in the interest of such simplification include the employment of the terms "circumenteric connectives" and "subenteric ganglia" for those portions of the nervous system for which the currently used term includes "-oesophageal" or "-pharyngeal," depending upon the detailed spatial relationship of the digestive system to the nervous system in the particular animal. This attempt to simplify the terminology of invertebrate morphology is, in the opinion of the reviewer, a splendid start for a much needed program.

Volume II includes all the acoelomate Bilateria. The bulk (407 pp.) of this volume is given over to the Platyhelminthes; the last 73 pages are devoted to the Rhynchocoela or Nemertina. Volume III deals with all the pseudo-coelomate Bilateria. The Acanthocephala are allowed 52 pages, the Aschelminthes 468 pages, and the Entoprocta 34 pages.

Probably the phylum Aschelminthes will be the most controversial major feature in the classificatory scheme set forth by Dr. Hyman in these two volumes. This grouping-together of the Rotifera, Gastrotricha, Kinorhyncha, Priapulida, Nematoda, and Nematomorpha will be considered by many to be an unjustified combination. This phylum is essentially the older Nemathelminthes of the last century, but the more recent name, "Aschelminthes," proposed by Grobben has been preferred, inasmuch as it has not had historically the great variety of meanings that the former has had. With respect to the grouping of the six classes in the single phylum, Hyman states: "The alternatives in regard to these groups would seem to be to elevate each to the rank of a separate phylum, or to associate them loosely under one phylum. Those who prefer the first alternative should not hesitate to adopt it. The first alternative is accepted here because. . . . " A rather good case is eventually made for the loose grouping by pointing out evidence indicating that each of the six groups is, in one way or another, more or less closely related to each of the others. Some

[1] New York: McGraw-Hill Book Co., Inc., 1951. Vol. II, pp. 550; Vol. III, pp. 572. $9.00 each.

[2] The last ones were *Cambridge Natural History* (1895–1909) and Lankester's *A Treatise on Zoölogy* (1900–1909).

may be surprised to find the Priapulida included in this assemblage. But after reviewing the evidence it appears remarkable that this particular disposition of this hitherto controversial group had not been made earlier. Indicative of the objective manner in which Dr. Hyman reached these conclusions are the facts that, at the time of preparation of Volume I, the Acanthocephala were to be considered a class of the Aschelminthes and the Priapulids were to be considered Eucoelomates.

The general organization of the chapters is quite similar to that of Volume I—in fact, the chapter numbers are continuous with that volume. Each chapter commences with historical considerations, which are usually followed by a general definition and classification of the group. Each of those phyla having no classes—the Rhynchocoela, Acanthocephala, and Entoprocta—has a general treatment of the morphology and physiology, including reproduction and development, of the group as a whole and a final section on phylogenetic considerations. The chapters on the Platyhelminthes and the Aschelminthes are subdivided to treat the classes one by one.

The reviewer is very glad to see the pseudocoelomate Entoprocta removed from their wholly artificial but usual association with the eucoelomate Ectoprocta with which they are still so frequently considered as a single phylum, Bryozoa or Polyzoa.

Both volumes are copiously illustrated, Volume II containing 208 figures and Volume III, 223; the majority of these figures are full-page ones and include a number of items. The vast majority of the illustrations have been prepared by the author herself and are well done. Both volumes are provided at the end of each chapter with extensive bibliographies, which are subdivided into sections to parallel the taxonomic sections into which the text is divided. Altogether, in the two volumes there are 94 pages of bibliography, which are estimated to include between four thousand and five thousand individual references. The indexes are adequate, though one might wish that they were somewhat more detailed.

As general reference works, these volumes will undoubtedly become indispensable to virtually every zoölogist. There is nothing comparable to them in English at present, nor is there likely to be for many years to come, in view of the tremendous amount of work which must go into the preparation of such compendiums and the very few American biologists who might be both qualified and free to attempt such an extensive project.

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